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LABORATORY REPORT No. 304

BIOCHEMICAL PROCEDURES
VOLUME I

by

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CHEMISTRY DIVISION

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US ARMY MEDICAL RESEARCH
AND NUTRITION LABORATORY

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BIOCHEMICAL PROCEDURES -- VOLUME I

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GENERAL PROCEDURE FOR PROCESSING ANALYTICAL SAMPLES

1. The most important factor in achieving reliable analytical results is obtaining a truly representative portion of the whole sample. To assure that the aliquot removed from a sample represents the original material, the following steps should be taken:

a. Warm all stored homogenates to 37° C. and reblend in a Waring blendor type homogenizer.

b. Unless specifically instructed otherwise, adjust all urine samples to a pH of 3.5 with hydrochloric acid. Warm all stored urine samples to 37° C. and agitate to dissolve as much sediment as possible.

c. Warm stored sera to 37° C. and centrifuge to remove fibrin or extraneous growth. If an analysis for total protein is to be performed, check with supervisor on condition of samples.

2. Only methods designated as acceptable by the supervisor will be used. No modifications may be applied to any procedure without approval of the Branch Chief.

3. Use both internal and external standards. For all automated procedures these standards should be interspersed with the unknown samples.

4. Place all data in loose leaf notebooks on the work sheets provided for the particular determination. Fill in all the information on the work sheet. Note any discrepancies in samples or procedure.

5. All calculations shall be verified by both the analyst and another individual and so initialed on the work sheet. The calculation rechecks shall be performed beginning with the basic data.

6. All work sheets will be given to the supervisor on completion. Absolutely no data will be released to any other individual. All analytical results will be processed through the supervisor who will recheck the data, prepare reports for the principal investigator and log and record the data in appropriate ledgers and file folders.

THE USE OF STANDARDS IN LABORATORY CONTROL

Types of Standards:

1. Reference or primary standard: a substance containing the element, functional group or compound to be analyzed (the analyte) and having the following properties:

   a. Established purity, preferably with a certified analysis.

   b. Completely soluble in the media used in the procedure.

   c. Inert to normal changes in pH and temperature employed in the method.

   d. Stable for reasonable period of time, either when refrigerated or frozen.

2. Pooled standard: a substance similar in composition to the material being analyzed and having the determined element, compound or functional group present in a range of concentration similar to the unknown.

   a. Blood serum or plasma, urine and well-homogenized material may be used as pooled standards.

   b. The pooled specimen is analyzed 15 or more times, depending on the accuracy of the method and the homogeneity of the sample. This average value becomes a control value which is used in subsequent analyses to evaluate the reproducibility and reliability of the method. The pooled specimen is subdivided into aliquots suitable for triplicate analysis. These aliquots are placed in ampules and sealed or put in screw cap vials. Each is suitably identified by content and expiration date.

3. Internal standard: a substance, usually the reference standard, used to validate results.

   a. This material is analyzed as an additional sample with the unknowns or added in known amounts to an unknown which has been previously analyzed.

   b. This standard provides a check on recovery and validity of sampling procedures.

The Use of Standards in Laboratory Control

Use of Standards:

1. Reference or primary standard: The reference material is used to obtain a standard curve, graph or equation which provides a basis for calculating unknown values.

2. The pooled standard provides a means of obtaining repetitive checks on instrument and method reliability based on average pool values.

3. The internal standard enables the analyst to check on the validity of the sample results by calculating per cent recoveries of standard added to known samples.

Application of Standards as Control Measures:

1. All analytical determinations should be performed in duplicate, or triplicate, if either the methodology or greater precision warrants closer control.

2. Both internal and external standards should be used; the former being added to the sample and the latter being analyzed separately. No exceptions to this will be allowed.

ASH

Reference: Official Methods of Analysis of the AOAC. Ed. 9, 1960 (22.010)

A. Reagents: None

B. Procedure:

1. Place crucibles from moisture determination into a muffle furnace preheated to 550° C.

2. Allow samples to ash for 4 hours or overnight.

3. Remove crucibles directly to a vacuum dessicator to cool for 30 minutes.

4. Weigh crucibles to 0.1 mg for ash weight.

C. Notes:

1. Samples should be quickly put into the hot oven and the door closed. When the door is open, the samples will burst into flames with the possibility of losing part of the sample.

D. Calculation:

$$\% \text{ Ash} = \frac{\text{Ash wt.}}{\text{sample wt. (wet)}} \times 100$$

Express to 0.01 per cent.

MOISTURE

Reference: Official Methods of Analysis of the AOAC. Ed. 9, 1960 (22.003)

A. Reagents: None

B. Procedure:

1. Tare porcelain crucibles to the nearest 0.1 mg, in duplicate. Use existing markings on crucible for identification, making certain not to have identical markings.

2. Weigh samples of about 2 grams dry material to 0.1 mg. (For most homogenates of one part sample to two parts water, the sample weight should be about 5 grams.)

3. Place samples in a 95-100° C. vacuum oven at 15 pounds vacuum or greater for overnight drying. Set clock timer to shut off oven current at 0400 hours (4 a.m.).

4. Remove crucibles to vacuum dessicator to cool for 30 minutes under vacuum.

5. Weigh crucibles to 0.1 mg for moisture content.

6. Samples are now ready for ashing.

C. Notes:

1. Samples with very high moisture content or low ash values can be in error considerably from oils on the hands. It is best to wipe the crucibles when taring and handle them as little as possible.

2. Homogenates evaporate quickly in this dry climate. As a result, samples should be weighed quickly after they are put into the crucibles.

D. Calculations:

1. Moisture wt. = tare + sample (wet) - tare + sample (dry)

2. $$\% \text{ Moisture} = \frac{\text{moisture wt.}}{\text{sample wt. (wet)}} \times 100$$

Express to the nearest 0.01 per cent.

CRUDE FAT

Reference: Official Methods of Analysis of the AOAC. Ed. 9, 1960 (22 035)

Principle: A homogenized sample is hydrolyzed with 6 N hydrochloric acid in a boiling water bath. The crude fat is then extracted from the digested sample with an ether mixture, dried and weighed.

A. Reagents:

1. Hydrochloric acid (50%)
2. Ethanol (95%)
3. Ethyl ether (anhydrous) analytical reagent
4. Petroleum ether (boiling range, 30-60° C.) analytical reagent

B. Procedure:

1. Acid hydrolysis

a. Weigh to the nearest 0.1 mg, 3 to 5 grams of sample homogenate (about 2 grams of dry sample) using a large tip pipette to introduce the sample into the bottom bulb of a Mojonnier flask (see note #1).

b. Add 15 ml of 50% HCl to the flask and shake well to mix, being careful not to splash the sample up the flask.

c. Heat samples in a steam bath (90-95° C) for 90 minutes, shaking gently several times.

d. Remove flasks from bath and cool to room temperature.

e. Add 95% ethanol to just below the top of the lower constriction.

2. Ether extraction

a. Add 25 ml of ethyl ether, stopper with rubber stoppers and shake vigorously for several seconds.

b. Allow a minute to settle and gently remove stoppers and rinse (into the flask) with petroleum ether.

c. Add 25 ml of petroleum ether, stopper and shake on a mechanical shaker for 5 minutes.

d. Allow layers to separate, to clear and pour through filter paper into 250 ml glass extraction flasks. (Be careful not to pour over the lower portion.)

REPEAT THE FOLLOWING STEP THREE TIMES:

e. Add 50 ml of a 1:1 mixture of ethyl ether:petroleum ether, stopper and mechanically shake for 5 minutes very briskly. Allow layers to separate and again decant ether layer as before, rinsing the stoppers with petroleum ether.

f. Rinse filter paper with petroleum ether (5-10 ml) and discard the paper. Rinse the funnels down so they may be used again.

g. Evaporate the ether slowly on a steam bath. Remove immediately when dry. (Sometimes the fats will be oils; therefore, "dry" will mean the absence of ether fumes.)

h. Redissolve the fat in 25 ml of ether mixture and pour through new filter paper into preweighed, 150 ml fat flasks, tared to 0.1 mg.

i. Rinse the 250 ml flask three times, each with 1˘ ml of ether mixture and add to the filter. Finally, rinse the paper and funnel well with about 5 ml of the ether.

j. Evaporate slowly on a steam bath and remove immediately when dry (absence of ether fumes).

k. Transfer flasks to 95-100° C. vacuum oven for 3 hours at 15 pounds vacuum or greater. If left overnight, set clock to turn oven off after 3 hours.

l. Remove flasks, allow to cool if still warm and weigh to nearest 0.1 mg.

C. Notes:

1. If sample homogenates are not very liquid, add 2 ml of 95% ethanol to prevent clumping when the HCl is added.

2. Long exposure to air and temperatures over 100° C are to be avoided to prevent oxidation or destruction of short chain fats. If the samples must be left for 20 minutes or more, they must be stoppered.

3. Extreme care must be taken in pouring off the ether extracts to prevent pouring over the sediment. Even more important is careful pouring of the first extracts and the first transfer solutions. These are the most concentrated, and the loss of even a drop can appreciably effect the duplication.

D. Calculation

$$\% \text{ Fat} = \frac{\text{Fat wt.}}{\text{sample wt.}} \times 100$$

Express to 0.01 per cent

KJELDAHL PROCESS

Principle: Various nitrogenous compounds in biological materials are converted into ammonium sulfate by boiling with concentrated sulfuric acid in the presence of a suitable catalyst, e.g., mercuric oxide. Subsequently, the ammonium sulfate is decomposed by means of a fixed alkali (NaOH) and the liberated ammonia is collected in a boric acid solution. The collected distillate is titrated with sulfuric acid of known strength, and the nitrogen content of the sample under examination is computed.

A. Reagents:

1. Concentrated sulfuric acid, reagent grade.

2. Saturated sodium hydroxide: Fill an eight liter stainless steel pail 2/3 full with caustic soda flakes. Add tap water very slowly to the pail with constant mixing from the bottom up. Fill the pail to within 3 inches of the top. Continue mixing until dissolved. Allow to cool for 24 hours in a hood. Add sodium thiosulfate in the proportion of 25 gm per liter of caustic. The sodium thiosulfate is first dissolved in 1 liter of tap water. At this time, the specific gravity of the solution should fall in the range of 1.36 to 1.45. If above 1.45, add tap water until the proper value is obtained. If below 1.36, add more caustic soda to the solution.

3. 4% boric acid: 40 gm boric acid plus 960 ml distilled water. To each liter of boric acid, add 17.8 ml of mixed indicator.

4. Standardized sulfuric acid: 0.1 N and 0.4 N.

5. Catalyst: 42 gm red mercuric oxide and 600 gm anhydrous potassium sulfate ($\frac{1}{2}$ powdered + $\frac{1}{2}$ crystal, if available). These two compounds must be obtained as chemically pure, finely divided chemicals and mixed thoroughly to give a homogeneous preparation.

6. Mixed indicator:

a. 0.2 Bromcresol Green: 200 mg bromcresol green in 2.9 ml 0.05 N NaOH, which is mixed and ground in a mortar. Dilute to 100 ml with 95% ethanol.

b. 0.2% Methyl Red: 200 mg methyl red in 7.4 ml 0.05 N NaOH, which is mixed and ground in a mortar. Dilute to 100 ml with 95% ethanol.

c. The indicators are mixed in the proportions of 1 part 0.2% methyl red + 4 parts of 0.2% bromcresol green.

B. Procedure:

1. Sample preparation (all samples are set up in duplicate):

Urine:	2-5 ml	
Stool:	lyophilized:	0.3-0.8 gm
	wet:	1 - 3 gm
Diet:	lyophilized:	0.3-0.8 gm
	wet:	1 - 3 gm

a. Weigh solid sample on Glassine powder paper.

b. Weigh wet sample in a small beaker.

c. Record digestion flask number on report sheet.

d. Place powder paper and sample into the digestion flask or, in the case of liquids, rinse the sample into the digestion flask.

e. Add 6-10 glass beads to act as bubbling stones.

f. Add a level teaspoonful of catalyst.

g. Carefully add 25 ml concentrated sulfuric acid.

2. Sample digestion:

a. Place the digestion flask on the electric heaters.

b. Turn on the exhaust fans.

c. Allow the samples to digest for 1 hour after sample clears.

d. Turn the heaters off and allow the digestion flasks to cool until a white precipitate forms. DO NOT TURN THE EXHAUST FAN OFF.

e. If the sample solution in the digestion flask has a yellow color, digestion is not complete. Digestion for another hour is necessary.

3. Ammonia distillation:

a. Be sure that the sample solution is cool.

b. Carefully add 285 ml of distilled water.

c. Agitate to dissolve all the white precipitate.

d. Add a level teaspconful of talc to act as an anti-bumping agent.

e. Again, allow to cool.

f. Into a 400 ml beaker put 50 ml of 4% boric acid (containing indicator).

g. Insert the glass delivery tube into a beaker below the liquid level of the boric acid.

h. Turn on the water to the condensing apparatus. Temperature should be around 60-65° F. on the overhead thermometer.

i. Turn on the overhead exhaust fan.

j. Turn on the electric heaters to the third notch.

k. BE SURE THAT YOU ARE WEARING THE SAFETY GOGGLES AND PLASTIC APRON.

l. Carefully add 75 ml saturated sodium hydroxide to the digested sample solution. Allow the sodium hydroxide solution to run down the side of the digestion flask slowly, AVOIDING any agitation.

m. Insert the rubber stopper of the condensing apparatus into the neck of the digestion flask and swirl the flask very slowly.

n. After the color of the boric acid solution turns blue, collect about 75 ml of distillate (1/4").

o. Be sure to record the beaker number on the report sheet so that you can tell which beaker corresponds to which flask.

p. Lower the beaker to the stone ledge and allow 12 drops of distillate to fall into the beaker ($\frac{1}{2}$ - 1 minute).

q. Turn off the heat. If the heat is turned off before lowering the beakers, the distillate will be sucked back into the digestion flask and the sample will be lost.

r. Allow the distillate to cool.

4. Titration of distillate:

a. Using sulfuric acid of known concentration, titrate the collected distillate until its color changes from blue to a very light pink. Be very careful not to pass the end-point. For urine samples containing low levels of nitrogen, use standard sulfuric acid of about 0.1 N. For other samples, standard sulfuric acid of about 0.4 N can be used. Sulfuric acid solutions used in these titrations must be standardized to the fourth decimal place.

b. Acceptable variation between duplicates is 5% or less of their average value.

5. Calculations:

$$\frac{0.014 \times (\text{ml titration} - \text{ml blank}) \times \text{normality sulfuric acid} \times 100}{\text{volume in ml or weight in gm of sample}} = \%\ \text{nitrogen}$$

Conversion factors for converting % nitrogen to % protein are as follows:

Meats, fish, vegetables, fruits, composites, feces: 6.25

Bread, cereals, baked cereal products: 5.70

Milk, dairy products: 6.38

Report % nitrogen to the third decimal place and % protein values to the second decimal place.

6. Considerations for standardized procedure:

a. All analyses must be performed in duplicate.

b. Standard solutions must be analyzed daily as a check on the method.

c. When any reagent solution is freshly prepared, a reagent blank analysis must be performed. This is done by substituting 1 gm sucrose for the sample.

7. Precautions:

a. Caution should be used at all times with the Kjeldahl apparatus.

b. Keep acetic acid on hand to neutralize any saturated sodium hydroxide which may be spilled.

c. Wear goggles and apron at all times.

C. Determination of Normality: H_2SO_4 (two methods)

1. Use of NaOH of known normality (0.10 N).

a. Formula: $V_1 N_1 = V_2 N_2$

b. Volume_1: Volume of NaOH

c. Normality_1: Known NaOH

d. Volume_2: ml of H_2SO_4 used in titration

e. Normality_2: Calculated from above

2. Use of primary star ' d.

a. Accurately weig.)ut 0.2-0.5 gm dry sodium carbonate.

b. Transfer to a 250 ml flask.

c. Dissolve in 25 ml distilled water.

d. Add one drop phenolphthalein.

e. Titrate with the acid until just colorless.

f. Calculation:

$$\frac{\dfrac{\text{gm } Na_2CO_3}{106}}{\text{ml titration}} \times 1000 = \underline{N} \text{ of acid}$$

D. References:

1. J. Biol. Chem. 176: 1401, 1948.

2. Hawk, P. B., B. L. Oser and W. H. Summerson. "Practical Physiological Chemistry," pp. 874-876, The Blakiston Co. Inc., New York, 1954.

PROCEDURE FOR IN VITRO UTILIZATION OF ^{14}C

1. Tissue is weighed and put in a center well flask. Prefolded 2-cm square filter paper is placed in the center well. Flask is then capped with a rubber stopper. Tissue is incubated in a metabolic shaking incubator at 37° C for 3 hours.

2. At end of incubation period, 0.1 ml of 25% KOH is injected into the center well, saturating the filter paper, and 0.5 ml of 2 N H_2SO_4 is injected into the buffer. Incubation is then continued for 20 minutes.

3. At the end of the 20-minute period, the rubber stoppers are removed and the filter paper is placed directly into scintillation vials. Tissue is rinsed in normal saline three times, blotting on filter paper after each rinse, then put into saponification tubes containing 15 ml 2:1 chloroform/methanol.

Preparation of Fractions for Counting Radioactivity:

1. Filter Paper - $^{14}CO_2$: Let scintillation vials containing filter paper stand overnight to dry. Using applicator sticks, spread filter paper on bottom of vials. Add 10 ml of toluene. For the blank, use clean filter paper and 10 ml toluene.

2. Glycogen: Tubes containing tissue are stoppered (ground glass) and placed on shaking machine for 3 hours. Using tissue forceps, squeeze out excess chloroform/methanol and put tissue into screw-capped tubes containing 2 ml of 25% KOH. Heat tubes in boiling water bath a few minutes to dissolve tissue. Add 1 ml H_2O and 9 ml of 95% ethanol. Heat a few seconds to warm tubes with caps on. Do not boil. Put in refrigerator overnight to precipitate glycogen. Centrifuge for 10 minutes at 1000 rpm. Discard supernatant and invert tube and allow to drain for 5 minutes. Add 4 drops of 1 N H_2SO_4 to hydrolyze. "Mash" the sample with a stirring rod. Add 2 ml H_2O to tube, rinsing stirring rod. Mix and transfer 1 ml to scintillation vial. Add 10 ml Bray's solution. For the blank, use 1 ml H_2O and 10 ml Bray's solution.

3. Non-saponifiable Fraction: To saponification tubes containing lipid extract, add 4 ml of lipid salty wash (0.05% $CaCl_2$ = 250 mg in 500 ml H_2O),

mix on Vortex and let stand until two phases separate. Upper phase is discarded. Add 5 ml lipid salty wash, mix, allow two phases to separate. Upper phase is again discarded. Put 1-2 boiling chips into tubes. Boil to dryness in hot water bath or evaporate under a stream of nitrogen. Add 6 ml of 3% KOH in methanol (3 g KOH + 5 ml H_2O + 95 ml methanol). Saponify for 30 minutes at 80-85° C. Add 6 ml H_2O to each tube and cool. Extract three times with 5-ml portions of petroleum ether, transferring each petroleum ether fruction to a saponification tube. Add 10 ml H_2O to tubes containing ether. Mix. Transfer ether layer to scintillation vials. Add 5 ml ether to saponification tubes. Mix. Transfer ether layer. Evaporate petroleum ether and add 10 ml toluene scintillant. Blank is 10 ml toluene.

4. Fatty Acids: Saponification tubes are acidified with concentrated HCl. Check each tube with Congo Red Paper. Extract three times with 5-ml portions of petroleum ether, transferring each portion to a scintillation vial. Evaporate petroleum ether and add 10 ml toluene scintillant. Blank is 10 ml toluene.

5. Glyceride-glycerol: Transfer aqueous layer to a 25-ml graduated cylinder. Measure and record volume. Transfer 0.2 ml to a scintillation vial and add 10 ml toluene scintillant. Blank is 0.2 ml H_2O and 10 ml toluene.

When only the non-saponifiable and fatty acid fractions are desired, proceed as follows:

1. Following incubation, the tissue is rinsed as described in 3 above and placed in a saponification tube containing 10 ml 5% KOH in 95% ethanol.

2. Saponification is carried out for 30 minutes (adipose tissue) or 3 hours (liver) at 80-85° C. Following saponification, add 6 ml H_2O and cool.

3. Extract the non-saponifiable and fatty acid fractions as described in 3 and 4 above.

ISOLATION OF CHOLESTEROL DIGITONIDE FOR SCINTILLATION COUNTING

Reference: Kabara, J. J. J. Lab. Clin. Med. 50: 146, 1950.

Reagents:

1. Acetone:alcohol (1:1).
2. Acetone:ether (1:1).
3. Digitonin solution, 1%. (in 50% ethanol containing 0.1% acetic acid)*

Procedure:

1. Transfer non-saponifiable fraction to 15-ml conical centrifuge tubes and evaporate to dryness.
2. Dissolve in 4 ml of acetone:alcohol.
3. Add 1 ml digitonin solution.
4. Mix on Vortex mixer, wash sides of tube with small amount of acetone: alcohol.
5. Stopper and let stand overnight.
6. Centrifuge at 2500-3000 rpm for 15 minutes.
7. CAREFULLY decant and discard supernatant.
8. Wash with about 5 ml acetone:ether, mix on Vortex and wash sides of tube with acetone:ether.
9. Repeat centrifugation and decant supernatant.
10. Dissolve precipitate in exactly 1.5 ml of glacial acetic acid, warming tubes if necessary.
11. Transfer 1.0 ml to scintillation vial and add 10 ml of toluene scintillant, mix well and stopper. 1 ml acetic acid + 10 ml toluene scintillant is the blank.
12. Use 0.2 ml for colorimetric cholesterol determination (pipette 0.5 ml and 1.0 ml standards, evaporate to dryness and dissolve residue in 0.2 ml of glacial acetic acid. Add 0.2 ml of glacial acetic acid to blank tube).

*In a 100-ml volumetric flask, dissolve 1 g digitonin in 50 ml absolute ethanol; add 1 ml of 10% acetic acid; add distilled water to the 100-ml mark and mix.

Isolation of Cholesterol Digitonide for Scintillation Counting

Calculations:

$$\text{DPM} \times 1.5 = \text{DPM/sample}$$

$$\frac{\text{Cholesterol}}{\text{mg/sample}} = \frac{\text{Conc. of std.}}{\text{OD std.}} \times \text{OD unknown} \times 7.5$$

$$\text{Specific activity} = \frac{\text{DPM/sample}}{\text{mg/sample}} = \frac{\text{DPM}}{\text{mg}}$$

THE INCORPORATION OF PALMITATE-1-^{14}C IN TRIGLYCERIDE BY ADIPOSE TISSUE IN VITRO

Reference: Borgstrom, B. Acta Physiol. Scand. 25: 111, 1952.

Procedure:

1. Epididymal fat pads are taken from male mice, cut transversely into two pieces, weighed on a rapid weighing torsion balance and placed in a 20-ml beaker, containing 3.0 ml of buffer.

2. Krebs-Ringer phosphate buffer, pH 7.4, modified as described previously, is enriched with 3% B.S.A. and palmitate-1-^{14}C.

3. The tissue is incubated for 3 hours at 38° C in a Dubnoff metabolic shaker, at a shaking rate of 90 strokes per minute.

4. After incubation, the tissue is removed from the buffer, washed twice in 0.9% NaCl, blotted dry and placed in 5.0 ml of Dole's extraction mixture (4.0 vol. isopropyl alcohol, 1.0 vol. of heptane and 0.1 vol. 1 N H_2SO_4). The tissue is extracted for 12 hours.

5. After extraction, the tissue is homogenized in a ground glass homogenization tube and the contents washed into a separatory funnel with 5.0 cc of heptane.

6. The homogenate is then washed with 0.1 N NaOH (50% ethanol) to remove any free fatty acids, and the volume of the heptane fraction remaining after washing is measured and an aliquot transferred to a counting vial containing toluene and flours, for counting.

PROCEDURE FOR PERIODATE OXIDATION OF GLYCERIDE-GLYCEROL AND ISOLATION OF α-CARBONS AS THE DIMEDON DERIVATIVE

Principle:

Glycerol is oxidized to formaldehyde and formic acid by periodate. Formaldehyde is derived from the α-carbons and formic acid from the β-carbon. Dimedon will form a precipitate with formaldehyde which is soluble in toluene.

Reagents:

Periodic acid - (0.35 M) - 8 g/100 ml.

Dimedon reagent - (0.3 M) - dissolve 4.2 g 5,5-dimethyl-1,3-cyclohexanedione in 50 ml of ethanol, adjust volume to 100 ml with H_2O.

Toluene - spectro grade.

Procedure:

1. Aqueous fraction containing glyceride-glycerol (~12 ml) is desalted on a column of Amberlite IR 120 (H^+ form) and IR 45 (OH^- form), about 2 ml of each. The column is washed with H_2O.

2. The desalted solution is evaporated to dryness under reduced pressure and the residue dissolved in 9 ml of H_2O.

3. Eight ml of the solution is transferred to a glass stoppered tube and 0.5 ml to a scintillation vial for counting; 10 ml of Bray's solution is added to the vial for counting.

4. To the glass stoppered tube containing 8 ml of the glycerol solution is added 1 ml of freshly prepared periodic acid solution. The tube is mixed, placed in the dark and oxidation allowed to proceed for at least 12 hours.

5. The contents of the tube are neutralized with NaOH to pH 6.5-6.8. It is important that the solution be slightly acidic.

6. One ml of freshly prepared dimedon is added to each tube; the tubes are mixed and the formaldehyde derivative is allowed to form for at least 2 hours.

7. Five ml of toluene are added to each tube; the tubes are shaken vigorously, then centrifuged at low speed for about 10 minutes.

8. Three ml of the toluene layer are transferred to a scintillation vial and 10 ml of toluene scintillant solution added.

Procedure for Periodate Oxidation of Glyceride-Glycerol and Isolation of α-Carbons as the Dimedon Derivative

Calculation.

Total dpm in sample = dpm for 0.5 ml aliquot x 18

dpm in α-carbons = dpm in 3 ml toluene x 5/3 x 9/8

$$\%\ \text{radioactivity in } \alpha\text{-carbons} = \frac{\text{dpm in } \alpha\text{-carbons}}{\text{Total dpm}} \times 100$$

% radioactivity in β-carbon = 100 - % radioactivity in α-carbons

DECARBOXYLATION OF LONG CHAIN FATTY ACIDS

Reference. Brady, R. O., R. M. Bardley and E. G. Trams. J. Biol. Chem. 235, 3093, 1960

Principle.

Decarboxylation of long chain fatty acids is accomplished via the Schmidt Reaction.

$$R\text{-}\overset{\overset{\displaystyle O}{\|}}{C}\text{-}OH + HN_3 \xrightarrow{H_2SO_4} R\text{-}NH_2 + CO_2 + N_2$$

Reagents

1. Sodium azide.
2. Acid mixture -- one part fuming H_2SO_4 and three parts concentrated H_2SO_4.
3. CO_2 trapping mixture -- ethanolamire/methyl cellosolve, 1:2 (v/v).

Procedure

Pipette 1 ml of fatty acid solution into a scintillation vial and dry. Pipette 3 ml of same solution into a 10 ml reaction flask and evaporate to dryness under a stream of nitrogen. Approximately 50 mg of sodium azide are added and the flask is then covered with a Kontes septum stopper. The flask and entire collection apparatus is then flushed continuously with nitrogen gas. Be sure that bubbles appear in trapping mixture to ensure that the system is functioning properly, then inject 3 ml of acid mixture. Shake the tube for 1 hour at 70° C.

The evolved CO_2 is continuously trapped in 10 ml of trapping mixture according to the method of Baggiolini and Bickel (1) with the exception that the evolved CO_2 is "carried" into the trapping mixture with nitrogen rather than "pulling" it through under a vacuum.

Add 10 ml of Toluene scintillator to each vial containing dried fatty acids, and to each vial containing 10 ml of CO_2 trapping mix plus trapped CO_2. Count.

Decarboxylation of Long Chain Fatty Acids

Calculations:

F. A. dpm:

$$\frac{\text{cpm}}{\text{ml}} \times 3\ \text{ml} \times \frac{1}{\%\ \text{efficiency}} = \text{total dpm in F.A}$$

$deCO_2$ dpm:

$$\frac{\text{cpm}}{\%\ \text{efficiency}} = \text{total dpm in carboxyl carbon}$$

% ^{14}C in carboxyl carbon:

$$\frac{\text{Total dpm in carboxyl carbon}}{\text{Total dpm in F.A.}} \times 100$$

Reference.

1 Baggiolini, M. and M. H. Bickel. Anal. Biochem. 14: 290, 1966.

IN VITRO BUFFER MEDIUM FOR THE STUDIES OF THE METABOLISM OF ADIPOSE TISSUE, MUSCLE SLICES AND LIVER SLICES

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1 Umbreit W. W., R. H. Burris and J. F. Stauffer, Manometric Techniques, Burgess Publishing Co., Minneapolis, Minnesota 1959.

2 Fritz I. B., D. G. Davis, R. H. Holtrop and H. Dundee. Am. J. Physiol. 194: 379, 1958

Krebs Ringer Buffers

10.0 ml NaCl	4.5%	
0.4 ml KCl	5.75%	
0.014 ml $CaCl_2$	0.33%	48.0 ml H_2O
0.1 ml KH_2PO_4	10.55%	
0.01 ml $MgSO_4$	0.060%	

2.0 ml phosphate buffer pH 7.4 (0.5 M Na_2HPO_4 - HCl)

The above concentrations are 5 times that suggested by Krebs. The more concentrated solutions remain stable for longer periods. Upon preparation, the salt solution is diluted 1:5 and the pH adjusted to 7.4.

To the final buffer, 3% B.S.A. (bovine serum albumin) is added along with the substrate to be employed in the determination.

LIVER EXTRACTION

Principle A small sample of tissue is completely macerated in methanol with a Servall Omnimixer. The lipid fraction is then extracted in chloroform-methanol while heating to about 60° C

Reagents

Chloroform methanol, 3:2 (v/v) Reagent grade.

Procedure

1 Weigh approximately 1 gram of tissue to the nearest 0.1 mg (fresh tissue or frozen sample after thawing)

2 Homogenize in a minimum of methanol (~ 5-10 ml) until thoroughly macerated in a Servall Omnimixer

3 Transfer to 125-ml Erlenmeyer, washing cutter blades and cup several times with methanol to a total volume of about 20-25 ml.

4 Add chloroform to give approximately 3:2 ratio of chloroform:methanol (~ 35-40 ml $CHCl_3$)

5 Heat at 45-50° C in a water bath for 30 minutes.

6 Remove and allow to cool to room temperature.

7 Filter into 250-ml Erlenmeyer flasks, using 12.5 cm diameter fluted filter paper (Eaton-Dikeman Co. grade 512) to remove the non-lipid material.

8 Rinse flasks three times with chloroform, adding to funnel each time.

9 Rinse the filter paper twice with chloroform, remove filter paper and rinse funnel once

10 Evaporate samples to dryness under a moderate flow of oxygen-free nitrogen (temp ~60-65° C).

11 At this point, tare 50-ml Erlenmeyer flasks to the nearest 0.1 mg. Prepare funnels with Whatman #1 filter paper (7.0 cm diameter), each containing a spatula full of anhydrous sodium sulfate. Caution must be taken to insure that absolutely no sulfate falls into the flasks (the sodium sulfate removes any moisture remaining in the extract)

Liver Extraction

12. Add a small amount of chloroform (~ 10 ml) to the flask and a little sodium sulfate and mix well. Transfer to the filter paper followed by three washes of the flask. Rinse paper and funnel as before.

13. Evaporate the extract to dryness in a water bath under oxygen-free nitrogen (temp ~ 60-70° C).

14. Place dry samples in a dessicator until a constant weight is obtained. The samples are generally left overnight.

15. Weigh the flasks again to the nearest 0.1 mg to obtain the fat or lipid weight.

16. Add 25 ml of chloroform and mix well to redissolve the lipid material.

17. Pipette the following aliquots for the various analyses:

a. Total cholesterol - 0.5 ml to colorimeter tubes.

b. Lipid phosphorus - 1.0 ml to 25-ml graduated Pyrex tubes.

c. Glycerides - 3.0 ml to 15-ml graduated centrifuge tubes.

PLASMA EXTRACTION

Principle: An aliquot of plasma is extracted with chloroform:methanol while being heated. After centrifuging, aliquots of the clear supernatant are taken for the various analyses

Reagents:

Chloroform:methanol (3:2, v/v): Reagent grade.

Procedure:

1. Pipette 0.4 ml of plasma into a 15-ml graduated centrifuge tube.

2. With a constant delivery syringe, add 9.6 ml chloroform:methanol (3:2, v/v) with enough force to thoroughly mix the contents.

3. Heat the tubes in a 50° C water bath for 20 minutes.

4. Remove tubes from bath and allow to cool to room temperature.

5. Adjust volume to 10-ml mark with methanol.

6. Mix contents thoroughly (Vortex) and centrifuge at 2000 rpm for approximately 10 minutes.

7. Pipette the clear supernatant liquid for the various analyses as shown below:

   a. Total cholesterol - 1.0 ml to colorimeter tube.

   b. Total lipids - 1.0 ml to colorimeter tube.

   c. Lipid phosphorus } 6.0 ml to 15-ml centrifuge tubes.

   d. Glycerides } 6.0 ml to 15-ml centrifuge tubes.

8. Evaporate the 6.0-ml aliquot dry in water bath under O_2-free nitrogen. When cooled, add 6.0 ml chloroform.

9. Mix well to dissolve what residue is soluble.

10. Pipette 2.0 ml of extract to 25-ml graduated Pyrex tubes for lipid phosphorus.

11. The remaining 4 ml are used for glyceride determination.

Plasma Extraction

Notes:

1. Cooling to room temperature following the 50° water bath is essential to prevent excess evaporation and to maintain accuracy of the dilution.

2. Thorough mixing is necessary after addition of the methanol to ensure a completely homogenous mixture.

3. The chloroform:methanol has been shown to carry over a significant amount of inorganic phosphorus, making necessary a chloroform transfer for lipid phosphorus and glyceride determination.

FATTY ACID WORKUP

Important Precautions

1. Nitrogen protection at all times.

2. All solvents must be redistilled.

Procedure

1 Using 1-2 ml serum, place in 40-50 glass stopper centrifuge tube. Add 20 times vol. of serum of $CHCl_3$:MeOH (2:1). Mix with swirling before stoppering. Put on shaker for at least 15 minutes.

2 Add 1 ml MeOH for each 10 ml solvent used, mix and centrifuge. Pour supernatant in 125-ml Erlenmeyer flask and treat residue twice more with 5 ml $CHCl_3$:MeOH. Combine supernatants and bring to dryness, using stream of N_2 and warm H_2O bath (use hot tap H_2O).

3 Extract with petroleum ether (30-60°), add small amount of Na_2SO_4. Decant into small tube or flask. Reduce volume under N_2 to less than 0.5 ml.

4 Sample may be saponified for total fatty acids at this step or separated into groups by TLC.

5 For TLC. Sample is spotted on silica gel plate, also a set of standards at edge of plate and run in petroleum ether (30-60° C), ethyl ether and acetic acid (90:10:1 v/v). Solvent system may be varied to achieve maximum separation. Dry plate at room temperature and spray one set of standards with 0.2% 2,7-dichlorofluorescein in ethanol. Scrape off corresponding spots onto glassine paper and transfer into 15-ml centrifuge tubes.

6 Extract cholesterol ester and triglyceride zones with $CHCl_3$:MeOH (2:1) and the phospholipids with 10% formic acid in MeOH. Extract each sample first with 10 ml, then three times with 5 ml of the solvent.*

7 Combine supernatants and concentrate using N_2 and warm water bath. Sample may be stored in a small amount of petroleum ether in the freezer at this point before proceeding with the saponification.

*If total fraction is to be saponified and immediately, it is not necessary to extract the lipid fraction. The silica gel and sample may be saponified as outlined. The major portion of the silica gel is lost in the water wash of the saponification step.

FREE FATTY ACIDS

Reference. Dole, V. P. and H. Meinertz. J. Biol. Chem. 235: 2595, 1960.

Reagents (Keep all reagents flushed with nitrogen at all times.)

1. Indicator solution (thymol blue)

    a. Stock. 0.1% thymol blue in 95% ethanol.

    b. Working indicator. Pipette 10 ml of stock (a) into a 100 ml volumetric flask. Add 10 ml of distilled water and dilute to the mark with absolute ethanol. The final solution is 10% distilled water in ethanol.

2. Alkali titrant (approximately 0.018 N NaOH)

    a. Prepare a saturated solution of NaOH and allow 2 weeks for the carbonates to settle out.

    b. Accurately pipette 0.1 ml of the clear supernatant (saturated solution) into a 100 ml volumetric flask. (Be certain to wipe the excess from the outside of the pipette completely with a wet gauze moistened with distilled water, then dry.)

    c. Dilute to 100 ml mark with distilled water.

    d. Flush upper space in the flask with nitrogen, stopper and mix. Always flush with nitrogen after each use before stoppering.

3. Extraction mixture

a. Sulfuric acid (1 N H_2SO_4)	10 ml	or	20 ml
b. n-heptane	100 ml	or	200 ml
c. Isopropyl alcohol	400 ml	or	800 ml

4. Palmitic acid reference solution (50 mg/liter in distilled water)

    This solution is made at 1/3 the normal concentration in plasma since the standard is taken in heptane (6 ml) rather than water as the samples are (2 ml).

    The exact weight of palmitic acid is used as the basis for the standard concentration.

Indicator Adjustment

The acidity of this solution is important. Adjust the pH to require 10 to 20 µl of titrant to neutralize 1 ml of indicator under 3 ml of blank solution. (Use 0.01 N HCl or titrant for the adjustments.) The endpoint is a good blue which requires a little practice to reproduce.

Free Fatty Acids

Single Extraction Method:

1. Pipette 2 ml of fresh plasma into a glass stoppered tube (50 ml).

2. Add 10 ml of extraction mixture, stopper, and shake well and allow to stand about 5 minutes until layers separate.

3. Add. water - 4 ml distilled water
heptane - 6 ml (Shake again and allow to separate.)

4. Pipette duplicate 3 ml aliquots of the upper phase into 15 ml conical centrifuge tubes and stopper. (See NOTE #1)

BLANK: (two extractions with two aliquots from each = 4 titrations)

1. 2 ml distilled water.
2. 10 ml extraction mixture; shake well.
3. 4 ml distilled water.
4. 6 ml heptane; shake again and allow to separate.

STANDARDS: (two extractions with two aliquots from each = 4 titrations)

1. 2 ml distilled water.
2. 10 ml extraction mixture; shake well.
3. 4 ml distilled water
4. 6 ml standard solution in heptane; shake and allow to separate.

Fatty Acid Titration:

1. (a) Always keep distilled water in the burette when not being used for a period of time. (b) When finished titrating, empty burette of alkali, rinse once with distilled water and refill with distilled water. (When ready to use, empty distilled water, fill with base, empty, refill, empty and refill to about 1/4 inch below the zero mark.

2. Pipette accurately (wiping outside of pipette) 1.0 ml of indicator solution into 3 ml extract in the 15 ml centrifuge tubes and cork until ready to titrate. (Two layers form, clear on top and red to orange on the bottom.)

Free Fatty Acids

3. Place tube to titrate in the moving arm of the rack. With all valves closed, place the bubbling tube into the solution. Slowly open the main tank valve, then slowly open the regulator valve to get good mixing of the two layers. (Keep the bubbler well wiped before placing in each tube.)

4. Adjust titrant in burette to zero mark and wipe off the excess from the tip.

5. Raise the tube rack so the tip of the burette is just barely within the solution.

6. Add titrant slowly, raising bubbler out of the solution and allowing layers to separate when noting color of the lower layer.

7. The color changes from red to yellowat pH range 1.2-2.8 and from yellow to green to blue at pH 8.0-9.6 With practice, the endpoint is reproducible to about 1-2 µl of alkali*.

*Near the endpoint the color changes of y-yg-g-bg-b requires about 1 microliter per step.

Calculations.

The reference standard is 50 mg/liter palmitic acid, which is about 1/3 the concentration of NEFA in plasma. It is assigned 3 times this value to allow for the difference in volume of sample and standards taken.

$[\mathrm{STND}]$ = 50 mg/liter, and assigned 3 times this value = 150 mg/liter.

Palmitic acid M.W. = 256.42.

Therefore, $[\mathrm{STND}] = \dfrac{150\ \mathrm{mg}}{256.42}$ = 0.585 mM = 585 µMoles/liter.

This value is approximate, depending on the actual weight of the palmitic acid per liter.

$$\frac{[\mathrm{STND}]}{(\mu l)} = \frac{[\mathrm{Sample}]}{(\mu l)}$$

$$[\mathrm{Sample}] = [\mathrm{STND}] \times \frac{(\mu l)\ \text{sample titer}}{(\mu l)\ \text{stnd titer}}$$

$$\frac{[\mathrm{STND}]\ \mu M/\text{liter}}{(\mu l)\ \text{stnd titer}} \times (\mu l)\ \text{sample titer}$$

(Subtract the average blank reading from all samples and standard readings before calculating results.)

Free Fatty Acids

Notes:

1. The samples are kept on ice during the extraction. When received from the Ward, shake each tube vigorously several seconds and allow to separate. Transfer with disposable pipettes, the entire upper layer to small test tubes, cork and keep cool until used.

2. Normal range: Dole gives a value of about 585 μM/liter as an approximate value for plasma (J. Biol. Chem. 235: 2598, 1960).

SAPONIFICATION AND ESTERIFICATION OF LIPID MATERIALS

Important Precautions:

1. Nitrogen protection at all times.

2. All solvents must be redistilled.

Procedure:

1. Samples are transferred to saponification tubes (135 x 20 mm, § 19/38) and solvent removed using nitrogen and a warm water bath. To each tube 3 ml 0.5 N alcoholic potassium hydroxide and one boiling chip is added, samples refluxed for 30 minutes using water condensers and a water bath of approximately 70°. Additional boiling chips may be needed to ensure refluxing. Six samples may be done at one time.

2. The sample is cooled and transferred with water to a 125-ml separatory funnel to which has been added 1.5 ml 1 N hydrochloric acid and 15 ml ethyl ether. Stopper separatory funnel and shake well, opening stopcock frequently to release pressure. At this point, check water layer using pH paper that solution is acidic; if necessary, add more HCl; water layer should be clear.

3. Two separatory funnels are used for each sample. The water layer from the above step is transferred to separatory funnel #2 to which 10 ml ether has been added. Add small amount of water to separatory funnel #1 to prevent loss of ether through stopcock. Sep. funnel #2 is shaken well and water layer transferred to a flask and the ether layers are combined in sep. funnel #1, rinsing sep. funnel #2 well with water which flows into sep. funnel #1 The ether layers are washed two times with water using the rinse of the sep. funnel #2 as one washing.

4. The second water washing is removed and a small amount of sodium sulfate is added to the sep. funnel. The ether is then transferred from the top of the sep. funnel into a large test tube (20 x 150 mm). The sep. funnel and sodium sulfate are rinsed once more with 10 ml ether and combined with the first ether solution. The sides of the test tube are rinsed and sample brought to dryness in a stream of nitrogen in a lukewarm water bath.

Saponification and Esterification of Lipid Materials

5. A boiling chip and 2 ml BF_3MeOH (12.5% w/v) solution is added to each tube and tube put into a hot water bath (approx. 70°) and refluxed 2 minutes (no cover). The time is started when the methanol begins to boil.

6. Using sep. funnel #2 from previous step (having rinsed it well with distilled water) 30 ml of water are added to the sep. funnel and 30 ml petroleum ether (30-60° C) are used to transfer the BF_3 mixture to the sep. funnel.

7. Contents of funnel are shaken well, allowed to separate and the lower water layer removed. The petroleum ether (top) layer is poured from the top into a 50-ml Erlenmeyer flask and a small amount of sodium sulfate is added. Flask flushed with nitrogen and stoppered. At this point, sample may be stored in the freezer. Petroleum ether is used for all rinsings in this step.

8. To proceed: Part of the solvent in the flask is removed in a nitrogen stream and the remainder transferred to a sublimation tube (135 x 20 mm, ₮ 19/38), rinsing the flask and sodium sulfate two times with small amounts of petroleum ether. Samples are brought to dryness in a warm water bath in a stream of nitrogen. The last few drops of petroleum ether are blown off while tube is rotated so that the sample is deposited evenly on sides of tube.

9. Tubes are placed on the manifold to which special Fisher-Nonaq stopcock grease has been applied. Start the vacuum pump after having filled the trap with dry ice and acetone. When vacuum is less than 0.05 mm, start the water in the cold fingers and immerse tubes in 60° water bath for 2 hours. Check vacuum several times in this 2-hour period.

10. Remove tubes and manifold from the water bath, turn stopcock slowly to release vacuum and rinse samples from cold fingers into special tubes (75 x 10 mm with ends pulled to a point for easy sample removal) using petroleum ether. Samples are now ready for the gas chromatograph and may be stored stoppered in freezer.

TOTAL PLASMA LIPIDS

Reference: Huerga, J. DeLa, C. Yesinick, H. Popper. Estimation of total serum lipids by a turbidimetric method. Am. J. Clin. Path. 23: 1163-1167, 1953.

Principle: The lipids when taken up in the p-dioxane are emulsified with the H_2SO_4 and form small droplets (about 10 microns in diameter) which are fairly uniform, even after 24 hours. The turbidity is measured with a spectrophotometer.

Reagents:

1. p-dioxane. Reagent grade.
2. Sulfuric acid solution. Dilute 40 ml conc. H_2SO_4 to 1 liter with distilled water (4%).

Procedure:

1. Evaporate the 1.0-ml aliquot to dryness under O_2-free nitrogen in a hot water bath (~70-80° C).
2. Remove the tubes and allow to cool.
3. Add 1.5 ml of p-dioxane to samples and a blank tube.
4. Heat tubes in boiling water bath for 1 minute.
5. Cool to room temperature.
6. Add 5.0 ml of sulfuric acid solution to samples and blank.
7. Mix well and allow to stand 30 minutes.
8. Read optical density at 650 mμ against reagent blank.

Calculations: The results are calculated from standard curves for the individual sera, human, chick or rat as the case may be. The curves are based on gravimetric lipid determinations. The equations are straight line functions which best fit the lines to each set of points and may be used in calculating the results.

NOTE: This particular procedure is not valid for liver extracts.

TOTAL CHOLESTEROL

Reference: Searcy, R. L. and L. Berquist. Clin. Chim. Acta 5: 192, 1960.

Principle: The chloroform:methanol extract is treated with ferrous sulfate. The addition of concentrated sulfuric acid produces a colored complex which can be quantitated spectrophotometrically.

Reagents:

1. $FeSO_4:CH_3COOH$: Sufficient ferrous sulfate ($FeSO_4 \cdot 7H_2O$), approximately 100 g/liter acid, is allowed to stand in glacial acetic acid for 1-2 hours. The solution is then filtered and is stable at room temperature for many months. If the solution has any color, it should not be used. Impure acid containing peroxide or oxidizing impurities will cause the solution to be yellowish-brown (see notes).

2. Concentrated H_2SO_4: Analytical reagent grade.

3. Cholesterol Standard: Dissolve 100 mg of C.P. cholesterol in cold chloroform (refrigerator temperature) and make to 100 ml. 10 ml of this solution diluted to 100 ml with cold chloroform yields a 0.1 mg per milliliter working standard. (All volumes and measurements should be made cold and quickly to prevent volume changes due to temperature change.)

Procedure:

1. Prepare standard tubes in duplicate, using same volume of standard as sample used, and a blank tube prepared likewise, using 3:2 chloroform:methanol or pure chloroform, depending upon the sample solutions.

2. To all the tubes add 6.0 ml $FeSO_4 - CH_3COOH$ reagent.

3. Add 2.0 ml conc. H_2SO_4 using a constant delivery syringe with enough force to mix contents.

4. Mix well (Vortex) and allow 10-15 minutes to cool.

5. Read optical density at 490 mμ against the blank.

Total Cholesterol

Calculations:

Dilution factor = 25

Stnd = Standard concentration (mg/ml)

OD_s = Standard optical density units

$OD_?$ = Sample optical density units

1. General Equation:

a. $\frac{[Stnd]}{OD_s}$ = mg substance/OD unit (OD scale calibration)

b. $\frac{[Stnd]}{OD_s} \times OD_?$ = mg substance in aliquot taken for colorimetry

c. $\frac{[Stnd]}{OD_s} \times OD_? \times$ Dilution Factor = mg substance/particular sample

2. Plasma: Results expressed in mg % or mg cholesterol/100 ml serum or plasma.

a. When 0.4 ml of plasma is diluted to 10 ml in the extraction procedure, 1 ml of the extract is equivalent to: $\frac{0.4}{10} = 0.04$ ml plasma.

b. To determine results for 1.0 ml plasma, the result must be multiplied by the dilution factor: $\frac{1.0}{0.04} = 25$.

c. The total plasma cholesterol then becomes:

mg chol./100 ml plasma = mg % = $\frac{[Stnd]}{OD_s} \times OD_? \times 25 \times 100$

If these exact volumes and dilutions are used, and using the standard concentration of 0.1 mg/ml, this equation can be simplified to the following:

$$\text{mg \%} = \frac{250}{OD_s} \times OD_?$$

d. If the sample requires a different dilution, the following formula must be applied:

$$\text{mg \%} \left(\frac{[Stnd]}{OD_s} \times \text{Dil. Fact.} \times 100\right) \times OD_?$$

Total Cholesterol

For a given set of data, the terms inclosed within the parentheses can be calculated separately as a factor (F) to simplify final calculations:

$$\text{mg \%} = F \times OD_?$$

3. Liver: Results on tissue samples are generally expressed as mg cholesterol per gram of sample tissue.

a. Samples of approximately 1 gram are usually extracted and the total fats made to a volume of 25 ml with chloroform. Taking 0.5 ml for cholesterol gives a dilution factor of $\frac{25}{0.5} = 50$.

b. From general equation "c":

$$\text{mg chol.}/\sim/\text{g} = \frac{[\text{Stnd}]}{OD_s} \times OD_? \times 50$$

Substituting for [Stnd] of 0.05 mg/0.5 ml and simplifying:

$$\text{mg chol.}/\sim/\text{g} = \frac{250}{OD_s} \times OD_?$$

c. This previous value is best calculated for all samples first, then divided by sample weight for reporting as:

$$\text{mg chol./g tissue} = \frac{\left(\frac{2.50}{OD_s} \times OD_?\right)}{\text{sample wt.}}$$

d. For samples requiring different sample weights and dilutions, the appropriate values must be used

Notes:

Occasionally, unexpected problems have arisen with the acetic acid reagent, resulting in variations in the OD readings. It would appear that even with reagent grade acetic acid and ferrous sulfate, occasionally, if the solution is allowed to stand overnight before filtering, some degree of oxidation takes place altering the color development. As a result, the solution has been allowed to stand 1 hour with frequent shaking and used either immediately or stored after filtering. This has appeared to eliminate this problem. No noticeable changes have been found after filtering the solution.

LIPID PHOSPHORUS

Reference: Taussky, H. H. and E. Shorr. A microcolorimetric method for the determination of inorganic phosphorus. J. Biol. Chem. 202: 675, 1953.

Principle: The lipid extract is digested with sulfuric acid and heated until the material chars. Hydrogen peroxide oxidizes the reduced inorganic phosphorus to phosphates which, in acid solution with the molybdate reagent, forms phosphomolybdic acid which, in turn, is reduced by ferrous sulfate to produce a blue colored complex.

Reagents:

1. 4.5 N Sulfuric Acid: Add 125 ml of concentrated H_2SO_4 to 500 ml of distilled water in a liter volumetric flask; mix, cool and dilute to the liter mark with distilled water.

2. 30% Hydrogen Peroxide: H_2O_2

3. Molybdic Acid Solution: Carefully add 83 ml of concentrated H_2SO_4 to 300 ml of distilled water in a liter volumetric flask. Mix and cool. Dissolve 50 g of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (ammonium molybdate) in 400 ml of distilled water in a beaker. Add molybdate solution to sulfuric acid; mix and dilute to the liter mark with distilled water.

4. Ferrous Sulfate Solution: Add 111 ml of 4.5 N H_2SO_4 to approximately 100 ml of distilled water in a 500-ml volumetric flask and mix. Add 62.5 g of reagent grade $FeSO_4 \cdot 7H_2O$ and shake well to dissolve. Adjust to 500 ml with distilled water (best made fresh weekly).

5. Ferrous Sulfate-Molybdic Acid Reagent: Mix just prior to use. In an amber reagent bottle, prepare sufficient reagent for number of samples according to the following proportions:

Molybdic acid solution	-	1 volume
Ferrous sulfate solution	-	2 volumes
Distilled water	-	2 volumes

Lipid Phosphorus

6. Standard:

a. Stock: Dissolve 526.4 mg of pure monopotassium phosphate in distilled water and adjust to 1 liter. Add 10 ml of chloroform as a preservative and refrigerate (0.12 mg P/ml).

b. Working Standard: Dilute stock standard 1 to 10. Concentration = 0.012 mg P/ml.

Procedure:

1. Evaporate the extract (1 ml for liver or 2 ml for plasma) to dryness under O_2-free nitrogen in a water bath (~80° C).

2. Add 1.0 ml of 4.5 N H_2SO_4 each to the samples, 1 ml of standard (in triplicate) and to a blank tube.

3. Heat the samples only at 200° C in heating block until white fumes appear and the sample is charred (about 20-30 minutes).

4. Cool and add 3-4 drops of 30% H_2O_2 and again heat until white fumes appear. One or two additional drops of peroxide may be necessary if the solution is not clear (about 15-20 minutes).

5. Cool and adjust volumes of samples, standards and blank to 6.0 ml with distilled water.

6. Add 4.0 ml of ferrous sulfate-molybdic acid reagent and mix.

7. Allow to stand 30 minutes (color is stable for at least 2 hours).

8. Read optical density at 660 mμ against reagent blank.

Notes:

1. The H_2SO_4 concentration is critical for proper color development in the given time. Therefore, all tubes (samples, standards and blank) should be treated identically with each reagent.

2. Care should be taken to ensure complete decomposition of the excess H_2O_2 during the second heating step.

Lipid Phosphorus

3. The color development is almost complete at 15 minutes and is very stable for 2 hours. Even at 4 hours there is only a slight increase. However, best results will be obtained if optical density is determined between 30 minutes and 2 hours.

4. The general equations introduced in the cholesterol calculations are basic here, also.

Calculations:

1. Plasma: Results expressed in mg %.

a. In the extraction, 0.4 ml plasma is diluted to 10 ml, and 2.0 ml taken for L.P. which is equivalent to 0.08 ml plasma $\left(\frac{0.4}{10} = 0.04 \text{ ml plasma/ml extract}\right)$.

b. To express results in mg/ml plasma, a dilution factor of 12.5 must be employed $\left(\frac{1.0}{0.08} = 12.5\right)$.

c. Total plasma lipid phosphorus then becomes:

$$\text{mg L.P./100 ml plasma} = \frac{[\text{STND}]}{OD_s} \times OD_? \times 12.5 \times 100$$

Using 1.0 ml L.P. standard (0.012 mg P/ml) and the above dilutions, this equation can be simplified to:

$$\text{mg\% P} = \frac{15}{OD_s} \times OD_?$$

d. Should different dilutions be required of a sample, the following calculation must be used:

$$\text{mg\% P} = \left(\frac{[\text{STND}]}{OD_s} \times \text{Dil. Fact.} \times 100\right) \times OD_?$$

For a given set of data, the terms in parentheses can be calculated separately as a factor (F) to simplify the calculations:

$$\text{mg\% P} = F \times OD_?$$

Lipid Phosphorus

2. Liver: Results expressed as mg lipid phosphorus per gram tissue.

a. Normally, lipid extracts are dissolved in 25 ml of chloroform; 1.0 ml is taken for lipid phosphorus (dil. fact. = 25).

b. From general equation #3 under CHOLESTEROL =

$$\text{mg L.P./particular sample} = \frac{[\text{STND}]}{OD_s} \times OD_? \times 25$$

Using 1.0 ml L.P. standard (0.012 mg P/ml) and simplifying:

$$\text{mg L.P./particular sample} = \frac{0.30}{OD_s} \times OD_?$$

c. For reporting as mg L.P. per gram liver, this value which should be calculated first for each sample, must be divided by sample weight:

$$\text{mg L.P./g tissue} = \frac{\left(\frac{0.30}{OD_s} \times OD_?\right)}{\text{sample wt.}}$$

d. For samples requiring different sample weights and dilutions, the appropriate values must be used.

GLYCERIDE DETERMINATION

References.

1. Leveille, G. A., J. W. Shockley and H. E. Sauberlich. U. S. Army Medical Research and Nutrition Laboratory Report #255, 1961.

2. Van Handel, E. and D. B. Zilversmit. J. Lab. Clin. Med. 50: 152, 1957.

Principle: Phospholipid free plasma extract is obtained by adsorption of the phospholipids onto FLORISIL. The samples are saponified with potassium hydroxide yielding glyceride-glycerol which is oxidized to formaldehyde by periodic acid and the reaction halted with sodium arsenite solution. Fatty acids and cholesterol are removed by chloroform extraction. The formaldehyde is reacted with chromotropic acid producing a color which can be quantitated.

Reagents:

1. FLORISIL Floridin Company, Tallahassee, Florida.

2. Chloroform. Reagent grade.

3. Alcoholic KOH (0.4%): Dissolve 2 g reagent grade KOH in 5 ml of distilled H_2O and dilute to 100 ml with pure ethanol (2% KOH). Dilute either (a) 1 to 5 with 95% ethanol; or (b) 10 ml of 2% KOH + 2 ml of distilled H_2O to 50 ml with pure ethanol. (Make fresh daily.)

4. Sodium metaperiodate (0.05 M): Dissolve 1.07 g of reagent grade $NaIO_4$ in distilled water in a 100-ml volumetric flask. Refrigerate.

5. Sodium arsenite (0.5 M). Dissolve 6.5 g of reagent grade $NaAsO_2$ in distilled H_2O in a 100-ml volumetric flask. Refrigerate.

6. Chromotropic acid (0.2%): Add 600 ml of concentrated H_2SO_4 carefully, while cooling in cold water, to 300 ml of distilled water. Dissolve 2.24 g of chromotropic acid (4.5-dihydroxy-2,7-naphthalene-disulfonic acid, disodium salt, Eastman Organic Chemicals, Rochester, New York) in 100 ml of distilled water; add cooled dilute sulfuric acid to chromotropic acid solution, mix well and store in a brown reagent bottle. Prepare fresh at least every 2 weeks and store in refrigerator

Glyceride Determination

7. Sulfuric acid (10 N): Add 28 ml of reagent grade sulfuric acid to 60 ml of distilled water; when cool, dilute to 100 ml with distilled water.

8. Glyceride standard: Dissolve 0.040 mM of glyceride (tripalmitin) to a final volume of 100 ml in cold, reagent grade chloroform. Store in refrigerator (4.0×10^{-4} mM/ml).

Procedure:

1. Add FLORISIL to the 0.5 ml mark on the 15-ml centrifuge tubes containing the extracted lipids in chloroform. For serum samples, an extract containing lipids from approximately 0.16 ml of serum is taken. For liver samples, an extract containing lipids from approximately 150 mg of tissue (wet weight).

2. Shake the tubes vigorously on a Vortex mixer for several minutes each to ensure as complete a L.P. removal as possible. Centrifuge for 10 minutes at 1500 rpm and pipette 3/4 of original chloroform volume to 15-ml graduated centrifuge tubes.

3. Evaporate under O_2-free nitrogen in hot water bath.

4. Add about 0.5-1.0 ml chloroform and wash down sides of tube and evaporate dry again.

5. The standards and blank are carried from this point on. Pipette 0.5 ml glyceride standard in triplicate.

6. To each tube add 1.0 ml of freshly prepared 0.4% ethanolic KOH and heat at 65° C in water bath for 30 minutes.

7. Cool, add 1 ml of distilled water and 4 drops of 10 N H_2SO_4. (The procedure may be halted at this point.)

8. Add 0.5 ml of sodium metaperiodate solution and shake intermittently for exactly 10 minutes.

9. Add 0.5 ml of sodium arsenite solution with occasional shaking, again for 10 minutes (a brown color appears after 10-15 seconds which eventually fades).

Glyceride Determination

10. Adjust volume to 5.0 ml with distilled water and mix.

11. Add 0.75 ml of chloroform and shake thoroughly (Vortex).

12. Centrifuge at 2000 rpm for 10 minutes.

13. Transfer 1 ml of clear supernatant to Pyrex test tubes.

(During the next two steps, the samples and chromotropic acid should be protected from direct light.)

14. Add 5.0 ml of chromotropic acid and mix well.

15. Cover tubes with clean glass marbles and heat in a boiling water bath for 30 minutes.

16. Cool tubes in a pan of tap water (about 10 minutes).

17. Transfer solutions to colorimeter tubes and determine optical density at 570 mμ.

Calculations:

1. Plasma:

$$\text{mM/100 ml} = \frac{2 \times 10^{-4}}{OD_{STND}} \times \text{OD Sample} \times \text{DIL. FACTOR*} \times 100$$

$$\mu\text{M/100 ml} = \frac{0.2}{OD_{STND}} \times \text{OD Sample} \times \text{DIL. FACTOR*} \times 100$$

*DILUTION FACTOR: 0.4 ml of plasma in 10 ml extract =

$\frac{0.4}{10} = 0.04$ ml plasma per ml of extract. Therefore:

$$\text{DIL. FACTOR} = \frac{1.00 \text{ ml}}{0.04 \times \text{vol. of extract taken (ml)}}$$

$$\text{For 3.0 ml of Extract: D.F.} = \frac{1.00}{0.04 \times 3} = \frac{1.00}{0.12} = 8.33$$

$$\mu\text{M/100 ml} = \frac{0.2}{OD_{STND}} \times \text{OD Sample} \times 8.33 \times 100$$

$$= \frac{0.2}{OD_{STND}} \times \text{OD Sample} \times 833$$

$$= \frac{167}{OD_{STND}} \times \text{OD Sample}$$

Glyceride Determination

2. Liver:

$$\mu M/g = \left(\frac{0.2}{OD_{STND}} \times \text{OD Sample} \times \text{DIL. FACT.} \right) \div \text{Sample weight}$$

$$= \frac{F}{\text{Sample Wt.}}$$

$$\text{DIL. FACT.} = \frac{\text{volume to which samples are made}}{\text{volume of extract taken}}$$

Notes:

1. With the resulting volume such (8-9 ml) after collecting sample and wash through tne columns, it is necessary to either evaporate the chloroform to approximately 0.5 ml, or to dryness, and add back 0.5 ml to wash down the residue remaining on the sides, for proper saponification by the ethanolic KOH.

2. The 10-minute reaction period for the metaperiodate is critical and should be closely adhered to.

3. Due to the inherent error in this method, utmost care should be taken throughout the procedure.

SAPONIFICATION AND ESTERIFICATION OF FATTY ACIDS BELOW C_{14}:0

ALL solvents must be redistilled.

Nitrogen protection.

1. Samples saponified as usual using 3 ml 0.5 N KOH and 1 boiling chip, refluxing for 30 minutes.

2. Cool and transfer to separatory funnel using a small amount of water to rinse the tube.

3. Extract 3 times with 5 to 10 ml of diethyl ether, discarding the ether layer each time and rinsing the separatory funnel with water. This step removes the unsaponifiables. Emulsions, if any, are broken with small amount of ethanol.

4. Add 1.5 ml 1 N HCl to separatory funnel with 10 ml diethyl ether and extract the water layer. Check to see that the water layer is acidic with pH paper (H_2O layer should be clear). Extract the water layer a second time with a small amount of diethyl ether and combine ether fractions, washing once with water.

5. Remove water layer, swirl flask to remove water clinging to the sides and remove this. Add small amount of Na_2SO_4 and pour from top into a 15-ml graduated centrifuge tube, rinsing separatory funnel once. Bring to dryness using nitrogen and warm water bath. Further treatment of sample is required if water is still present, either by adding a small amount of Na_2SO_4 to centrifuge tube and excess petroleum ether or by extracting with petroleum ether and removing solvent layer with pipette. Either step is repeated 3 times.

From this point, use no warm water baths or strong streams of nitrogen. Once the methyl esters are formed, the C_{12}:0 and below are volatile and considerable losses may occur even with the evaporation of solvent.

Esterification - Diazomethane (H. Schlenk, Anal. Chem. 32: 1412, 1960):

Apparatus: Two side arm test tubes (16 x 150 mm) with side arm of one extending to bottom of second tube, making a tight seal with a rubber stopper.

Reagents:

a. 2-(β-ethoxyethoxy) ethanol (Carbitol) - purified by heating 1 hour with 5% KOH, and then distillation at approximately 90° C and 12 mm of pressure. Stable in refrigerator.

b. N-methyl-N-nitroso-p-toluenesulfanamide (Diazald-Aldrich Chemical Co.). This must be recrystallized in ether before storage in refrigerator.

c. 6 g KOH in 10 ml water.

ALL preparations are to be done in the hood as diazomethane is toxic and explosive in concentrated amounts. To be prepared only as it is used, and any excess is destroyed by adding, dropwise, acetic acid until yellow color disappears.

1. To the first test tube, add 10 ml ether and nitrogen is bubbled through the ether, saturating the nitrogen stream.

2. To the second test tube, add 2.1 ml Carbitol, 3 ml KOH solution and 2.1 ml ether. The side arm is immersed into an open test tube containing ether in an ice bath. To start reaction, add a few small pieces (size - tip of spatula) of Diazald to tube #2. Close and start a slow stream of nitrogen through the system.

3. To methalyate each sample, immerse tip of tube #2 into the sample containing 0.5 ml 10% MeOH in ether in an ice bath and continue to slowly bubble the diazomethane through until the sample turns the slightest yellow (almost instantaneous reaction). Remove sample from the system, washing the tip with a small amount of ether.

4. Remove excess diazomethane and solvent with a slow stream of nitrogen and a room temperature water bath.

5. Sample can be stored in a small amount of low boiling petroleum ether (0.5 ml) and then for GLC, the solvent is evaporated with care and a drop or more of carbon disulfide is added which will allow the detection of the lower chain methyl esters.

POLYANION PROCEDURE FOR β-LIPOPROTEIN

Reagents:

1. Mepesulfate - 1% aqueous solution.
2. Calcium acetate - 12% aqueous solution.

Procedure:

1. To 1 volume of plasma or serum (usually 0.2 ml), add 0.5 volume of mepesulfate and 0.5 volume of calcium acetate solutions.
2. Mix and let stand 30 minutes.
3. Mix and fill 2 capillary tubes (1.3-1.5 mm x 75 mm).
4. Seal tubes, being careful to have a flat seal.
5. Centrifuge 5 minutes (microhematocrit centrifuge).
6. With stage micrometer on low power microscope, measure the total length of fluid and height of packed column of precipitate.

Calculation:

$$\frac{\text{Height precipitate column}}{\text{Total fluid length}} \times 200 = \%\ \beta\text{-lipoprotein}$$

DETERMINATION OF LIVER GLYCOGEN

References:

1. Methods in Enzymology, vol. III, pp. 3-4 and 34-35.
2. Hawk, Oser and Summerson Practical Physiological Chemistry, p. 1071.
3. Steiten, D. et al. J. Biol. Chem. 222: 587, 1956.

Reagents:

1. KOH - 30% in distilled H_2O.
2. Ethanol - 60 and 95%.
3. 0.6 N HCl.
4. Reagents and methods for glucose.
5. 5 N sodium hydroxide (20 g/100 ml).
6. 1 N sodium hydroxide (4 g/100 ml).

Procedure:

1. To a 50-ml centrifuge tube, add 6 ml of 30% KOH and heat in boiling H_2O bath.
2. Remove tissue and drop into hot KOH (1-3 g).
3. Cover the tube with marble and continue heating for 45 minutes with frequent agitation, particularly during the first 15 minutes.
4. Filter (while hot) through glass wool into 40-cc graduated centrifuge tube, rinsing with 1-ml aliquots of 30% KOH.
5. After tubes have cooled, add an equal volume of 95% ethanol with constant mixing.
6. Heat mixture with agitation until white precipitate forms but avoid boiling.
7. Let precipitate stand for at least 2 hours and preferably overnight (in refrigerator) for complete precipitation.
8. Centrifuge, decant and discard supernatant (leaves brownish-white precipitate).
9. Add 5 ml of 60% ETOH, mix with glass rod and rinse rod with small amount of 60% ETOH. Centrifuge and discard supernatant; repeat washing procedure.

Determination of Liver Glycogen

10. If precipitate is still brown or if decanted supernatant is not clear, add 1 ml of distilled H_2O, mix and add 2 ml of 95% ETOH; mix, centrifuge, decant and discard supernatant.

11. Let white precipitate dry by inversion or place in dessicator overnight.

12. Add 6 ml of 0.6 N HCl to the precipitate. Place marble over top of tube and heat for 2 hours in a boiling water bath to hydrolyze the glycogen.

13. Cool.

14. Transfer contents to 10-ml volumetric; filter if there is much debris.

15. Add 1 drop of thymol blue.

16. Add 5 N NaOH dropwise until color changes from red to yellow (if glucose will not be determined within 5 hours, stopper); when ready to proceed with glucose determination, add 4 N NaOH until blue color appears and add distilled H_2O to mark.

17. Carry out glucose determination and express values as g glucose per 100 g of tissue.

ASSAY OF α-GLYCEROPHOSPHATE DEHYDROGENASE ACTIVITY

Reference: Beisenherz, G., T. Bücher and K. Garbade. α-glycerophosphate dehydrogenase from rabbit muscle. Methods in Enzymol. 1: 391, 1955.

Reagents:

1. Glycyl-glycine buffer - 0.25 M, pH 7.4.
2. Substrate - dihydroxyacetone phosphate (0.025 M).
3. NADH - 0.2 mg/1.2 ml (5 mg/30 ml).

Assay:

1.	Tissue extract	0.5 ml
2.	NADH	1.2 ml
3.	Glycyl-glycine	0.5 ml
4.	H_2O	0.5 ml
5.	Substrate	0.1 ml

Rx is started with substrate and OD is monitored at 340 mμ.

Blank - omit substrate.

PREPARATION OF CHOLESTEROL EMULSION FOR INJECTION

Reference: Meier et al. J. Biol. Chem. 198: 105, 1952.

Procedure:

1. To dry cholesterol, add Tween 20 -- 1 drop per mg.
2. Add sufficient alcohol to dissolve cholesterol.
3. Evaporate alcohol under a stream of nitrogen.
4. Dilute viscous cholesterol-Tween solution to desired volume with sterile saline.
5. Check activity by counting an aliquot in Bray's solution.

DETERMINATION OF TISSUE GLYCOGEN WITH ANTHRONE REAGENT

Reference: Methods in Enzymology, vol. III, pp. 34-37, 1957.

Principle: The tissue is digested with hot 30% KOH. The glycogen is precipitated with ethanol. The glycogen is then determined as glucose with anthrone reagent.

Reagents:

1. 30% aqueous KOH.
2. 95% ethanol.
3. Concentrated H_2SO_4.
4. 0.2% anthrone reagent. Dissolve 0.2 g of anthrone in concentrated H_2SO_4. The reagent is not stable and should be prepared prior to use.
5. Glucose standards - stock standard 0.3% glucose in saturated benzoic acid solution. Prior to use make up standard solution containing 30 µg/ml as follows: Dilute 1 ml of stock standard to 100 ml, with distilled water.

Procedure:

1. Add 3 ml of 30% KOH to conical 15-ml centrifuge tubes. Just prior to use, place tubes in a boiling water bath and cover tubes with marbles.
2. Weigh a piece of tissue weighing approximately 1 g and drop into hot alkali. Replace marble.
3. Heat in boiling H_2O bath for 45 minutes with occasional shaking, particularly during the first 15 minutes.
4. After digestion is complete, cool tubes.
5. Precipitate by adding, with mixing, 3.5 ml of 95% ethanol.
6. Heat with agitation until precipitate forms, but avoid boiling.
7. Let precipitate stand for at least 2 hours and preferably overnight (in refrigerator) for complete precipitation.
8. Centrifuge the tubes. Decant and discard supernatant, then invert tubes over filter paper and allow to drain.
9. Dissolve glycogen in hot distilled water and transfer to 50-ml volumetric flasks, adjust to volume with distilled water and mix.

Determination of Tissue Glycogen with Anthrone Reagent

10. Transfer appropriate aliquots (containing 20-100 μg glycogen) to graduated pyrex tubes (0.2 ml for liver and 0.5 ml for muscle). Adjust volume to 5 ml with distilled water. Three standards containing 1, 3 and 5 ml, respectively, of diluted standard and a blank tube containing 5 ml of H_2O are included.

11. Place tubes in cold water bath and add carefully 10 ml of anthrone reagent. Mix.

12. Heat tubes in a boiling H_2O bath for 10 minutes.

13. After heating, place tubes in a cold water bath to cool.

14. After tubes have cooled, transfer contents to spectrophotometer tubes and read at 620 mμ after setting instrument to zero with blank tube.

15. If OD is greater than 1.00, rerun sample, using a greater dilution.

Calculations:

$$\text{μg glycogen in tissue used} = \left[\frac{30}{OD_{30}\ \text{μg std}} + \frac{90}{OD_{90}\ \text{μg std}} + \frac{150}{OD_{150}\ \text{μg std}} /3\right] \times \text{OD samp.} \times 0.9^{*} \times \text{dilut.}$$

*0.9 = factor for conversion of glucose to glycogen (an equal weight of glycogen will read 11% higher than glucose).

Dilution = Liver - made up to 50 ml 0.2 ml taken = 250

Muscle - made up to 50 ml 0.5 ml taken = 100

$$\text{Glycogen (\%)} = \frac{\text{μg in tissue}}{\text{wt. of tissue}} \times 1/10{,}000$$

PREPARATION OF PROTEIN-FREE FILTRATES

References:

1. Folin and Wu. J. Biol. Chem. 38: 81, 1919.

2. Hawk, Oser and Summerson. Practical Physiological Chemistry, 12th ed., 1949. The Blakiston Co., Philadelphia, p. 493.

Principle: Proteins are precipitated from plasma or serum by tungstic acid and removed by filtration or centrifugation.

Reagents:

1. Sodium tungstate, 10%. Dissolve 100 gm of reagent grade, carbonate-free sodium tungstate ($Na_2WO_4 \cdot 2H_2O$) in water and dilute to 1 liter. Stable indefinitely.

2. Two-thirds normal sulfuric acid. Weigh out 35 gm of concentrated H_2SO_4, dilute to 1 liter and mix. Check by titration against standard alkali.

Procedure:

1. Transfer a measured quantity of plasma or serum to a flask having a capacity at least 15 times that of the volume taken.
2. Add 8 volumes of water and mix.
3. Add ½ volume of 10% sodium tungstate solution and mix.
4. Add slowly and with shaking ½ volume of 2/3 N H_2SO_4; shake well.
5. Let stand 10 minutes.
6. Transfer to a centrifuge tube and centrifuge 10 minutes at 2500-3000 rpm.
7. The clear supernatant is a 1 : 10 protein-free filtrate of plasma or serum.

DEPROTEINIZATION PROCEDURES

1. Sulfosalicylic acid

a. Add 5 parts of a 3% aqueous sulfosalicylic acid solution to 1 part sample, centrifuge or filter.

b. Urinary mucoproteins will not precipitate by sulfosalicylic acid alone. Prepare the 3% sulfosalicylic acid solution to contain 1% picric acid where mucoproteins are suspected.

2. Picric acid

a. Deproteinize with 1% aqueous solution of picric acid. Remove resultant precipitate by centrifugation.

b. Excess picric acid is removed with a small column of Dowex 2-X10 resin, using 0.02 N HCl to wash column. Objection is that usually the dilution resulting is excessive and requires concentration by lyophilization.

3. Sodium tungstate

a. Prepare reagents as follows: 0.6 N H_2SO_4 (8.35 ml conc. H_2SO_4 diluted to 500 ml with distilled water) and 10% aqueous sodium tungstate.

b. For plasma: 1 ml plasma, 1.4 ml water, 0.35 ml 0.6 N H_2SO_4 and 0.25 ml 10% sodium tungstate.

c. Mix, let stand a few minutes and then centrifuge at 1800 rpm or thereabouts for 5-10 minutes; collect the supernatant (filter if necessary), store under few drops of toluene in refrigerator or freezer.

4. Ultracentrifugation - Spinco Model L preparative centrifuge: More time-consuming, but useful in certain special situations where the above procedures may cause destruction of exceedingly labile compounds, etc.

PLASMA AMINO ACIDS NITROGEN

References:

1. Frame et al. J. Biol. Chem. 149: 255, 1943.
2. Russell. J. Biol. Chem. 156: 467, 1944.

Principle: The amino acids in a protein-free filtrate of plasma are determined by means of the color produced when amino acids react with β-napthoquinone-4-sulfonic acid in an alkaline solution.

Reagents:

1. Amino acid standard solution: The glycine and glutamic acid standards are stable; the mixed standard should be freshly prepared.

   a. Glycine standard: 268 mg of glycine are dissolved in distilled water in a 500 ml volumetric flask. Add 35 ml of N HCl and 1 gm of sodium benzoate. Dilute to 500 ml.

   b. Glutamic acid standard: 525 mg of glutamic acid are dissolved in distilled water in a 500 ml flask. Add 35 ml of N HCl and 1 gm of sodium benzoate. Dilute to 500 ml.

   c. Mixed standard: Each stock standard contains 0.1 mg of amino acid nitrogen per ml; 3 ml each of the glycine and glutamic acid standards are diluted to 100 ml in a volumetric flask; this mixed standard contains 0.006 mg of amino acid nitrogen per ml.

2. 0.1 N sodium hydroxide solution: Prepare a N NaOH solution by dissolving 4.0 gm of reagent grade NaOH in water and dilute to 100 ml; dilute 10 ml of this to 100 ml to prepare 0.1 N NaOH.

3. Borax solution: Dissolve 10 gm of sodium tetraborate ($Na_2B_4O_7 \cdot 10\ H_2O$) in water and dilute to 1 liter. This solution is stable.

4. Napthoquinone solution: Dissolve 250 mg of Na β-napthoquinone-4-sulfonic acid in water and dilute to 50 ml. Prepare immediately before using and discard remaining solution.

5. Acid formaldehyde solution: To water in a 1 liter volumetric flask, add 25 ml of concentrated HCl and 3 ml of 40% formaldehyde and dilute to 1 liter (0.04 M formaldehyde in 0.30 N HCl).

Plasma Amino Acids Nitrogen

6. 0.05 N sodium thiosulfate: Dissolve 12.5 gm of crystalline sodium thiosulfate in water and dilute to 1 liter.

7. 0.25% phenolthalein solution. Dissolve 0.25 gm of phenolthalein in 95% ethanol and dilute to 100 ml with ethanol.

Procedure:

1. Transfer 2 ml of a 1:10 tungstic acid filtrate of whole blood or plasma to a graduated pyrex tube.
2. In two similar tubes, place 2 and 4 ml of mixed standard and in a third tube 4 ml of water; this tube will serve as a blank.
3. To each tube, add 1 drop of 0.25% alcoholic phenolthalein.
4. Add 0.1 N NaOH drop by drop until a permanent pink color is obtained.
5. Add water to adjust volume of all tubes to approximately 5 ml.
6. Add 1 ml of borax solution from a constant delivery syringe with enough force to mix.
7. Add 1 ml of freshly prepared napthoquinone solution; mix.
8. Place all tubes in a boiling H_2O bath and heat 10 minutes.
9. Cool tubes in cold water for 5 minutes.
10. Adjust volume of all tubes to 13 ml with water.
11. With a constant delivery syringe, add 1 ml of acid formaldehyde solution followed by 1 ml of 0.05 N sodium thiosulfate solution.
12. Mix by transferring to colorimeter tubes.
13. Let tubes stand at room temperature 10-20 minutes.
14. Read at 470 mμ, setting the instrument to zero with the blank tube.

Calculations:

$$\frac{\text{Conc std.}}{\text{O.D.std.}} \times 500 \times \text{OD unknown} = \text{amino acid N mg/100 ml of blood or plasma}$$

SERUM ELECTROPHORESIS

References:

1. Beckman Model R-101 Microzone Electrophoresis Cell, Preliminary Instruction Manual RM-IM-2, August 1963.

2. Beckman Technical Bulletin, RB-TB-004, August 1963.

Principle: This method utilizes a Beckman Microzone Electrophoresis Cell with a Spinco Duostat power supply for the serum component separation on cellulose acetate support membranes. The membranes are stained with Ponceau-S fixative dye, and the densities of the zones determined with a Spinco Analytrol fitted with a Beckman Microzone scanning attachment.

Reagents:

1. Buffer B-2 (barbital buffer, pH 8.6, ionic strength 0.075): Dissolve one packet of Beckman buffer B-2 powder in 1 liter of distilled water, rinsing packet thoroughly. Several hours may be required to dissolve if cold water is used. It can be made the day prior to use. Store in refrigerator overnight to prevent mold growth. (Four normal runs can be made before discarding and refilling the cell.) One liter lasts about a day and a half (3 refills of the cell).

2. Fixative Dye Solution: Dilute one 30-ml bottle of Beckman fixative dye solution to 250 ml with distilled water. The final solution yields the following percentages by weight:

   a. 0.2% - Ponceau-S stain.

   b. 3.0% - Trichloroacetic acid

   c. 3.0% - Sulfosalicylic acid.

   d. 93.8% - Distilled water.

At 100 ml per day, this lasts about 2 days (may be stored several days in a closed bottle)

3. Acetic Acid Rinse (5% by volume): Dilute analytical grade glacial acetic acid with distilled water as follows:

   a. 50 ml acid to 1,000 ml or

   b. 100 ml acid to 2,000 ml.

4. Alcohol Rinse (95% ethanol): Make up as needed from anhydrous ethanol. Dilute 50 ml anhydrous ethanol to 1 liter with distilled water. (Use a fresh 40 ml per tray with each cell refill.)

5. Clearing Solution (25% acetic acid in 95% ethanol): Make fresh with each cell refill, or twice daily, as follows:

a. 25 ml glacial acetic acid.

b. 75 ml 95% ethanol.

Procedure:

1. Preparation for Run:

a. Prepare reagents as above and pour the following amounts:

Tray #	Contents	Volume*
1	Buffer	40 ml
2	Fixative dye	100 ml
3	Acetic acid rinse	40 ml
4	Alcohol rinse	40 ml
5	Clearing solution	100 ml

*These volumes should be reasonably accurate for optimum conditions.

b. Make certain siphon tube in cell is dry and fill with buffer using a disposable dropper. Be certain all air bubbles are forced out. Fill both sides of the cell to the marks and replace cell cover and upper lid. Position membrane mount on top of a 250-ml beaker with hinged guides open.

c. Serum samples must be free of any fibrinogen. Place one drop of serum on parafilm on a hard surface and cover immediately with the sample covers.

d. Check the condition of the applicator tip (see RM-IM-2). The two ribbons should be parallel with no bowing.

2. Sample Application:

a. Remove a membrane from the box with the tweezers, pulling it across the pan lip, and let it gently fall onto the surface of the pre-buffer. It will curl up, then flatten out. When surface completely drakens, shake tray to cover. The entire process takes about 10-15 seconds. Do not soak membranes.

b. Lift the membrane from the tray and place between two blotters and blot once across the top of the blotter with the hand.

c. Place the membrane on the bridge so the two identification holes will correspond to position "1" on the cell cover. This is important to have the first sample applied in this position with the two holes for identification.

d. Close the hinged membrane guides and place the bridge in the cell and cover. (Again, make certain double holes correspond to #1 position.)

e. Connect cell to Duostat with the special cable but DO NOT TURN ON at this point. Allow two minutes to equilibrate.

f. To wash applicator tip (1) prior to use and (2) between samples, rinse with a firm stream of distilled water and blot on blotter three times.

g. Following a rinse, skim the tip across the surface of the sample drop several times, blot the tip to prevent diluting the sample with the rinse and reload. Repeat several times before applying sample to the membrane.

h. Place loaded applicator, with tip retracted, in positioning slots and press white release button. Allow about 10 seconds and press red retractor button.

i. Samples should be run in duplicate, 4 samples per membrane. (Positions 1 and 2 being one sample and its duplicate.)

j. Immediately after applying the last sample to the membrane, cover the cell and thoroughly rinse the applicator tip and blot dry.

3. Electrophoretic Run:

a. Initial Duostat settings:

Output selector - 200-500 (constant voltage).

Meter range - x 100 volts.

b. Turn output adjust on and position at about the 10 o'clock position until indicator swings across the scale and levels off. Adjust output to 250 volts (2.5 on the scale).

c. Start timing the run for 20 minutes.

d. Switch meter range to x10 MA and record initial current. Also, record final current immediately before turning off the current at the end of the run.

Current Range: Initial 3.5 - 5.8 ma

Final 4.5 - 9.5 ma

If these ranges are not observed during a run, check with the manual.

e. At the end of 20 minutes, turn output adjust to off.

4. Membrane-Fix and Dye:

a. Remove bridge to the rim of a beaker.

b. Transfer the membrane quickly to the dye tray. Allow 10 minutes for dyeing and shake occasionally.

c. Transfer membrane to 5% acetic acid rinse. Shake gently and pour acid off. Repeat this rinse for a total of four rinses, but do not pour the fourth off.

d. Transfer with tweezers to the alcohol rinse and agitate gently for 1 minute.

e. Transfer to a tray of clearing solution, which contains a glass drying plate, and agitate gently for 1 minute.

f. Lift the glass plate and membrane at one end, positioning the membrane on the plate with tweezers, and allow to drain as much as possible.

g. Lift onto a blotter and draw the rubber squeegee gently over the membrane to remove any pools of solution. (CAUTION: Use very little pressure or the membrane will distort.)

h. Place the membrane and plate in a 110° C ventilated oven for 15 minutes.

i. Remove from oven, cool and peel the membrane off the glass and mount in a plastic envelope. (May be scanned immediately or stored for later.)

5. Scanning the Membranes:

a. Turn on power and lamp switches 15 minutes prior to run to allow for warm up.

b. Fill the pens with two drops of ink. Do not fill too full. Check to ensure its flowing.

c. Raise pen guard and pull pen lift button and insert the chart paper.

d. Place membrane envelope in metal carriage as follows: With the clips toward the operator, insert the membrane with the first sample (with two holes) in the window with the albumin (dark band) peak to the right. Center the scan between the two tape strips, positioning the vertical line just at the left edge of the albumin band.

e. Turn the carriage around and the pattern should be centered between the tape strips.

f. Insert the carriage from the right with the arrow up, sliding it in until the arrow is just visible at the right edge. (The first sample pattern is now about 3/16 inch from the slit opening.)

6. Zeroing and Calibrating the Analytrol:

a. Turn on the pen switch.

b. With the calibrate knob (upper right front) in the extreme counter-clockwise position, set the recorder pen to the zero line with the zero adjust knob (right side). This sets the neutral filter.

c. Turn calibration knob (front) to extreme clockwise position and adjust to the calibration set point (7.5) with the cell potentiometer knob (back, upper right from the front).

d. Repeat steps b and c several times, as changing one affects the other.

e. Push the pen lift button to lower the pen at the calibration set point for a permanent record and return the calibration knob to the extreme left and the scan is ready to begin.

f. Setting the speed control knob on 80 will allow a sample and its duplicate on the same chart. Experimentation is necessary to position the scans on the chart. The paper stops at 22.7 cm; therefore, 0-11 and 11-22 are convenient divisions. The albumin peaks should start by the second centimeter to establish a baseline fore and aft.

g. Turn on the analytrol feed switch and the speed control switch and scan the strip.

Serum Electrophoresis

h. When the pen establishes a baseline and reaches 11 cm, turn off both feed switches, position next strip, rezero and calibrate the analytrol and scan the duplicate.

i. At the end of the second scan, turn both feed switches and the pen switch off, pull the pen lift button, remove the chart and go on to the next chart and scan.

Notes: In referring to the Manual RM-IM-2, the alcohol recommended (J. T. Baker Reagent Alcohol) is not absolute. The composition is 95% ethanol, 5% 2-propanol.

Calculations: (Ref. Model R, paper electrophoresis system, Instruction Manual RIM-5, Beckman, p. 35-36).

1. If the initial and final baselines are not level and equal, establish the base by drawing a line from the beginning of the curve to the end.

2. Mark the initial and final points of the trace (leave and return to baseline) and the lowest points between individual peaks.

3. Draw a thin vertical line down from these points until they cross the integrator tracing of sawteeth.

4. Count the number of sawteeth per peak (between its vertical boundary lines). The sawteeth are arranged in groups of 10. Estimate to the nearest 0.2 whole tooth.

5. Total the number of sawteeth for the entire trace. Dividing the teeth per peak by the total number will give the percentage of that component. (Each sawtooth represents 0.1 square centimeter beneath the curve.)

6. As a calculation check, the total of the percentages should not differ from 100% by more than 0.2% (99.8% - 100.2%).

7. If difficulties arise in the evaluation of the trace, refer to RIM-5, pages 35 and 36.

A RAPID, DIRECT METHOD FOR DETERMINING URINARY UREA

References:

Ormsby, A. A. J. Biol. Chem. 146: 595, 1942.

Barker, S. B. J. Biol. Chem. 152: 453, 1944.

Principle: Urea reacts with diacetyl monoxime to form a yellow color. The color is intensified by the addition of potassium persulfate and measured in the Coleman spectrophotometer.

Apparatus and Reagents:

1. Ordinary 13-15 x 150 mm pyrex test tubes.
2. A boiling water bath.
3. Coleman Jr. spectrophotometer.
4. Sulfuric acid - a 50% solution by volume. Cautiously add 500 ml of concentrated H_2SO_4 to 500 ml of H_2O. Cool, transfer to a 1000 ml volumetric flask and make up to volume.
5. Diacetyl monoxime - a 3% water solution. Keeps indefinitely in a refrigerator. Stable for 4 weeks at 30-40° C.
6. Potassium persulfate - a 1% aqueous solution. Stable for 8 weeks in a refrigerator. Also quite stable for 6 weeks at room temperature.
7. Urea standard. Dissolve 215 mg (=100 mg urea N) of urea in a liter of distilled water. Add a little $CHCl_3$ or toluene as a preservative. Do not use thymol.

Urea Standard Curve:

1. To a series of test tubes, add duplicate standard aliquots as follows: 0.1, 0.2, 0.4. These tubes will then contain 10, 20 and 40 gamma of urea nitrogen, respectively.
2. Bring all volumes to 2.0 ml with distilled water. Prepare a blank containing 2.0 ml of distilled water.
3. Add 0.25 ml of 3% oxime solution to each tube.
4. Add 4 ml of 50% sulfuric acid solution (syringe pipette) and mix the tubes thoroughly.
5. Cap the tubes with marbles and place them in a vigorously boiling water bath for exactly 10 minutes.

6. Remove the tubes from the bath and, without waiting for them to cool, add 0.25 ml of potassium persulfate to each tube. Each tube should be shaken immediately after the addition of the persulfate.

7. Leave the tubes at room temperature and read each tube 15 minutes after the addition of persulfate at 420 mμ.

Urine Analysis:

1. Use a 1.0 ml aliquot of a 1 : 100 dilution of urine.

2. If readings are off the curve, it is possible to add 6.5 ml of distilled water to the sample for reading and multiply the result by 2.

Comments:

1. A straight line relationship exists up to 125+ gamma of urea. Above this, the relationship is not linear.

2. Heating for longer than 10 minutes may produce an undesirably dark color. Tubes must be kept from direct sunlight.

3. The color fades after 15-20 minutes. After 30 minutes of standing, the color has faded about 5%.

4. A factor calculated in this laboratory was:

$$\text{O.D.} \times F = \text{mcg N/tube}$$

$$F = \frac{\text{mcg N/tube}}{\text{O.D.}}$$

$$F = \frac{40}{.340}$$

$$F = 118$$

GRAVIMETRIC DETERMINATION OF INORGANIC SULFATES IN URINE

References:

1. Textbook of Quantitative Inorganic Analysis. Koltoff and Sandell.
2. Quantitative Clinical Chemistry. Peters and Van Slyke.
3. Analytical Chemistry of the Manhattan Project. Rodden.

Reagents:

1. 5% $BaCl_2$.
2. Diluted HCl solution (4 vol. H_2O + 1 vol. HCl).
3. Approximately a 1 mg/ml Na_2SO_4 (A.R.) standard.
4. Use chemically pure reagents in distilled, deionized water throughout the analysis.

Procedure:

Filter about 60 ml of urine, well-shaken and at room temperature, through a #1 Whatman filter paper. Pipet a 25-ml aliquot into a 250-ml beaker containing 100 ml of H_2O and 10 ml of diluted HCl. If the urine contains a very low concentration of $SO_4^{=}$, use 50 ml of sample and 75 ml of water. Add 10 ml of $BaCl_2$ dropwise; the urine must not be disturbed while the $BaCl_2$ is added. At the end of an hour, stir well and filter the precipitate quantitatively with a #42 Whatman filter paper. Also, use a rubber policeman to remove any precipitate clinging to the walls of the beaker or the stirring rod. Wash the precipitate with at least 200 ml of water, pouring aliquots of water from the reaction beaker into the funnel. Then, place the filter paper containing the precipitate into a preweighed, preheated (800° C) porcelain crucible. Dry the contents at about 100° C.

After drying, place the crucible into a furnace at 300° C, leaving the door partly open. When the filter paper finishes smoking and is reduced to ash, close the furnace door and let the cricible heat at 800° C for about 2 hours.

Gravimetric Determination of Inorganic Sulfates in Urine

Calculations:

$$\frac{\text{Mol. wt. of } SO_4^{=}}{\text{Mol. wt. of } BaSO_4 \times 25 \text{ ml}} \times \text{wt. of } BaSO_4 \times \text{total volume of urine} = \frac{\text{gm } SO_4^{=}}{\text{total volume}}$$

or $.01646 \times \text{gm of } BaSO_4 \times \text{total volume of urine in ml} = \frac{\text{gm } SO_4^{=}}{\text{total volume}}$

INORGANIC SULFATES IN URINE[†]

Reagents:

1. Disodium ethylenediamine tetracetate (1% solution).
2. Glacial acetic acid (3 M acetate buffer, pH 3.0) - must be absolutely pH 3.0.
3. Absolute ethyl alcohol.
4. Barium chloranilate powder (available as Fisher Certified Reagent, especially for colorimetric analysis of sulfate).

Procedure:

1. To a test tube are added 4 ml of a 1% solution of disodium ethylenediamine tetracetate.

2. Two ml of fresh (or preserved and refrigerated) urine* at pH 4.0 are then added.

3. Four ml of a 3 M acetate buffer at pH 3.0 and 10 ml absolute ethyl alcohol are added, and 0.1 g barium chloranilate powder is added with a spatula. This is an excess and need not be accurately measured for each sample.

4. The tube is shaken well several times over the next 15 minutes, after which it is filtered through a Whatman #42 filter paper into matched cuvettes suitable for use in the spectrophotometer.

5. The optical density of the sample is read at a wavelength of 530 mμ against a blank, prepared as are the samples, except that 2 ml of distilled water are used in the place of the urine. A standard solution containing 40 mg% SO_4 as sodium sulfate (sodium sulfate as 40 mg% as sulfate ion) is included with each group of determinations, and unknown values calculated.

*Adjust urine to pH 4 before using in test.

[†]Reference: Wainer, A. and A. L. Koch. Anal. Biochem. 3: 457, 1962.

MAGNESIUM DETERMINATION OF ASHED SAMPLES

Reference: Czajkowska, H. M. and J. C. Rathbun. The determination of calcium and magnesium in blood using one indicator. Can. J. Biochem. Physiol. 37: 225-229, 1959.

Reagents:

1. Water: Doubly distilled water (all glass apparatus) was used for all reagents.

2. EDTA stock solution: The disodium salt of ethylenediamine-tetraacetic acid was dried at 110° C for 4 hours and placed in a dessicator overnight, and then 4.804 g of the salt were dissolved and made up to 1 liter in distilled H_2O.

3. EDTA working solution: 20 ml of stock solution is diluted to 100 ml with distilled H_2O.

4. Eriochrome black T stock solution: 100 mg of the dye is dissolved in 100 ml absolute methanol, and 4 ml of concentrated NH_4OH added.

5. Eriochrome black T buffer solution: (Must be made up immediately before use.) To 100 ml of distilled H_2O are added 1.0 ml monoethanolamine and 1.7 ml of Eriochrome black T stock solution.

6. Ammonium oxalate. 2 g of reagent grade ammonium oxalate monohydrate are dissolved in 98 ml of distilled H_2O.

Procedure:

1. All glassware must be cleaned in an acid bath for about one day. The glassware is then rinsed in doubly distilled water and dried in an oven.

2. Pipette the following into a small test tube:
   a. 0.2 ml sample (ashed).
   b. 0.3 ml ammonium oxalate solution.
   c. 0.1 ml ammonium hydroxide.

3. Cover the tube with Parafilm and vortex for 30 seconds.

4. Spin down the precipitate in a centrifuge at 2000 rpm for 5 minutes.

5. Pipette 0.2 ml of the supernatant into a cuvette (pipette tip must not touch precipitate in bottom of tube.

Magnesium Determination of Ashed Samples

6. Immediately before reading on the spectrophotometer, pipette 10 ml of Eriochrome black T buffer into the cuvette.

7. Zero the spectrophotometer at 660 mμ against a distilled water blank.

8. Add EDTA to the sample cuvette (from step 6) in increments, recording the volume added and the per cent transmission after each addition. The solution should be well mixed (Vortex) between each increment. As the end point is approached, the volume of the increment should be reduced. The first appearance of color stability, indicated by minimum change in per cent transmission, is taken as the end point.

9. Titrate a blank containing 10 ml of the Eriochrome black T buffer solution to determine the amount of magnesium in the buffer. This value should be subtracted from the sample value to obtain the true amount of magnesium in each sample.

Calculations:

20 μl of EDTA working solution = 1 μg Mg

DITHIZONE METHOD FOR THE DETERMINATION OF SMALL QUANTITIES OF ZINC IN URINE, FECES AND TISSUE SAMPLES (1)

References:

1. Vallee, B. L. and J. G. Gibson, II. An improved dithizone method for the determination of small quantities of zinc in blood and tissue samples. J. Biol. Chem. 176: 435, 1948.

2. Reitz, L. L., W. H. Smith and M. P. Plumlee. A simple, wet oxidation procedure for biological materials. Anal. Chem. 32: 1728, 1960.

Principle: At pH 5.5 and in the presence of a tartrate solution and complex-forming buffer, dithizone combines with zinc in stoichiometric proportions to form zinc dithizonate, but does not combine with other metals which may be present.

Apparatus:

1. 125-cc separatory funnels.
2. Small glass or polyethylene funnels.
3. 50-cc volumetric flasks.
4. 19 x 150 mm cuvettes (all must be same type, new and without scratch marks).
5. Funnel racks.
6. Medicine droppers.

Reagents: All reagents must be absolutely zinc free. The best grade of chemicals should be obtained, but even these may contain enough zinc to interfere with the results and will have to be purified as described below.

1. Diphenylthiocarbazone (Eastman Kodak): 100 mg dissolved in 1000 cc of carbon tetrachloride AR (CCl_4). Store in acid washed (described below) Pyrex container at 4° C. Container should be covered in aluminum foil or other material to prevent light contact with solution.

2. Buffer Solution:

   a. 556 g sodium thiosulfate anhydrous AR ($Na_2S_2O_3$).

   b. 90 g sodium acetate anhydrous AR (H_3COONa).

   c. 10 g potassium cyanide anhydrous AR (KCN).

   d. Dissolve above in 1000 cc distilled deionized water. Solution is then titrated with 15 N acetic acid to an approximate pH of 5.5 using methyl red as an indicator. A final adjustment is made to pH 5.5 with a sensitive pH meter.

e. Solution is made up to 2000 cc with deionized water in a volumetric flask. The buffer is then shaken with portions of the dithizone solution in either 100- or 200-cc separatory funnels until the dithizone remains a clear green. Discard the dithizone and transfer buffer to an acid washed container. Let stand overnight. Check pH before using. Must be pH 5.5.

3. Tartrate Solution: 20% solution of sodium-potassium tartrate ($NaKC_4H_4O_6 \cdot H_2O$) made with deionized water. Extract with dithizone as in (2).

4. 0.1 N NH_4OH AR

5. 0.1 N H_2SO_4 AR

6. 6 N NH_4OH AR

7. Methyl Red Indicator: 1:100 alcoholic solution.

Cleaning of Glassware: Pyrex glassware must be used throughout procedure; ordinary soft glass contains zinc. All glassware used in this procedure must be washed with soap and water, rinsed with distilled water and immersed in 2 N HNO_3 for a minimum of 6 hours, preferably overnight. On removal from the acid, the glassware should be rinsed several times with deionized water and dried in a 100° oven. Allow nothing to touch the parts of the glassware which will be in contact with the sample solution. Particular precaution must be observed to prevent contamination.

Wet Oxidation Procedure: (2)

1. All the quantities mentioned in the subsequent directions are for samples of approximately 5 grams dry weight. For other quantities, the proportions should be altered accordingly. If urine is to be ashed, a minimum of 25 cc should be taken.

2. Weigh the sample accurately into a 250-cc Erlenmeyer flask and add 35 cc of concentrated nitric acid. All the acid can be added at once unless the sample is such that it foams badly on the addition of the acid. In this case, add only about 5 cc of acid; allow this to react before adding the remainder. Set the

flask on a hot plate using the lowest temperature setting in a hood to digest very slowly for about 4 to 6 hours. At this stage of the digestion, the solution is a clear yellow and the initial volume is not markedly reduced.

3. In the case of urine, pipette an aliquot into a flask and continue as above. If it is a very large aliquot, 50-100 cc, allow it to evaporate in a vacuum oven at 60° C for about 36 hours, then begin the ashing procedure. Less time is consumed when this step is used.

4. Next, add 2 cc of concentrated sulfuric acid and return the flask to the hot plate to heat rapidly. If the sample has a tendency to "bump" or splatter during this step, lower the heat and cover the flask with a stemless funnel until the sample boils smoothly.

5. The sample needs no careful watching until the volume is well reduced and a frothing action sets in. Charring appears rather suddenly. If a number of samples are being run simultaneously, the charred sample can be removed from the heat at this point and the oxidation completed at a later time, if desired.

6. To the hot, charred sample, carefully add dropwise a digestion mixture consisting of two parts of 72% perchloric acid and one part of concentrated nitric acid. By adding the perchloric acid dropwise, the danger of violent explosion is eliminated and the material is oxidized quickly and completely. About 5 minutes are required for this step. The color of the mixture gradually turns from black to brown to colorless as the digestion mixture is added and heating is continued. Only 0.5 to 3.0 cc of the digestion mixture are necessary to clear the material.

7. When the sample is clear, heat it strongly at the fuming stage for an additional 10-15 minutes. Only the white or slightly yellow mineral residue and a small amount of sulfuric acid are left in the flask. Remove the sample from the heat, cool, and add about 10 cc of distilled water and 3.0 cc of concentrated hydrochloric acid. Boil the contents gently to accomplish solution and then pour into a volumetric flask of appropriate size.

Dithizone Method for the Determination of Small Quantities of Zinc in Urine, Feces and Tissue Samples

8. A blank on the reagents must be prepared by evaporating the same quantity of the acids as used in the unknown. This blank should be run with the same set of samples with which it was prepared. The Zn value found for the blank is subtracted from the value found for the samples.

Procedure:

1. Stopcocks for the funnels are greased with silicon. 2 cc of the tartrate solution, together with 2 drops of methyl red, are added to 5 cc of the acid-ash solution in the separatory funnel. The contents of the funnel are then titrated with NH_4OH and H_2SO_4 to pH 5.5, at which methyl red has a peach color. 50 cc of buffer are added, and the contents are allowed to stand until the color has completely faded. Dithizone in CCl_4 (about 10 cc of a 1 or 10 mg% solution, depending on the amount of zinc present) is added, and the funnel is shaken vigorously for about 2 minutes. The dithizone in CCl_4 solution is allowed to collect in the bottom of the funnel, the last drop is shaken down and the CCl_4 phase is drawn off into a 50-cc volumetric flask. This procedure is repeated until the dithizone in the funnel remains a clear green. The sample in the volumetric flask is brought to volume with CCl_4. Depending upon the quantity of excess dithizone present, the final color may be purple or have a greenish tinge.

Colorimetry:

1. Dithizone in CCl_4 has an absorption maximum at 620 mμ; zinc dithizonate has an absorption maximum at 540 mμ, but is transparent at 620 mμ. Readings are obtained from the extracted samples at both 540 mμ and 620 mμ with the Beckman Model B Spectrophotometer. If the samples are too concentrated to get an accurate reading, dilutions will have to be made to bring them into range.

2. The zinc content of samples is calculated from the equation

$$Z = \frac{\dfrac{L^{540} - L^{620}}{R} \times D \times K}{\dfrac{100}{V}}$$

Z = total zinc in micrograms in the 5 cc of sample.

L^{540} = optical density at 540 mμ.

L^{620} = optical density at 620 mμ.

R = the ratio of densities of dithizone in CCl_4 at 620 mμ and 540 mμ.

D = the dilution factor with relation to the original volume (V).

K = the calibration constant.

V = the volume in which all of the extracted zinc dithizonate is originally diluted.

3. R must be determined with each set of samples. This can be done by diluting varying amounts of the dithizone working solution in 50-cc volumetric flasks (3 or 4), taking readings same as samples and dividing the reading at 540 mμ into the reading at 620 mμ. This should give a close range of numbers, the average of which is used for R.

4. Calibration constant: Prepare a stock solution of zinc chloride by dissolving 10 mg of metallic zinc in concentrated HCl and making up to a final volume of 1000 cc. From this solution, a series of standards containing from 4 to 50 gamma is prepared. These standards are extracted as described above, except that they are not dry ashed. The total zinc dithizonate is made up initially to 50 cc. Further dilutions with CCl_4 are then made to obtain readings within the optimal range of the instrument. The value for K is calculated from the following equation:

$$K = \frac{2Z}{\text{Corrected } L^{540} \times D}$$

Table I is an example of data which could be obtained and how K is calculated from these data.

Dithizone Method for the Determination of Small Quantities of Zinc in Urine, Feces and Tissue Samples

TABLE I

Total Zn	Dilution Factor	L^{540}	L^{620}	$\frac{L^{620}}{R(4.65)}$	Corrected L^{540}	K
gamma						
4	0	0.254	0.305	0.066	0.198	40.2
10	2	0.321	0.385	0.083	0.238	42.1
20	3	0.382	0.274	0.058	0.324	41.1
30	4	0.453	0.280	0.062	0.393	38.2
40	5	0.462	0.242	0.052	0.410	39.0
50	5	0.553	0.211	0.045	0.508	39.5
Average						40.0

Precautions:

1. When diluting the dithizonate solutions to bring them into a good range for the instrument, use 25-cc acid washed, volumetric flasks

2. If, when diluting the extracted zinc dithizonate samples with CCl_4 the dithizonate seems to change color, this may mean that the CCl_4 is contaminated and will have to be redistilled in an acid washed Pyrex apparatus.

3. The dithizonate solutions should not stand over 30 minutes before readings are taken.

4. When adjusting the pH of the buffer to 5.5, use NaOH concentrate or 15 N acetic acid. Never go too far on the acid side to cause use of more than 5 cc of NaOH per 2000 cc of buffer.

5. The pH of the buffer solution will change on standing and should be used within a week after being made.

Dithizone Method for the Determination of Small Quantities of Zinc in Urine Feces and Tissue Samples

6. If, after adding the buffer to the titrated samples the color does not fade on standing, the buffer may have become weak and should be prepared again. These samples should then be discarded and retitrated.

7. If the colored samples are extracted with dithizone, the color will also be extracted and will affect the readings on the spectrophotometer.

RED CELL HEMOLYSIS TEST FOR THE MEASUREMENT OF NUTRITIONAL ADEQUACY OF VITAMIN E

References:

1. Gordon, H. H., H. M. Nitowsky and M. Cornblath. Studies of tocopherol deficiency in infants and children. 1. Hemolysis of erythrocytes in hydrogen peroxide. Am. J. Diseases Children 90: 669, 1955.

2. Union of Burma Nutrition Survey, p. 171, ICNND Report, May 1963.

3. Harris, P. L., E. G. Hardenbrook, R. P. Dean, E. R. Cusack and J. L. Jensen. Proc. Soc Exp. Biol. Med. 107: 381, 1961.

Principle: Red blood cells show a decreased resistance to hemolysis in vitamin E deficiency. The rate of hemolysis when red cells are treated with a weak solution of hydrogen peroxide can be used as an index of the vitamin E status of individuals.

Reagents:

1. Normal saline solution: 8.5 g sodium chloride dissolved in 1 liter of distilled water.

2. Citrate saline solution: 1 g of sodium citrate is dissolved in 50 ml of distilled water in a 100-ml volumetric flask; make up to volume with double saline solution.

3. Phosphate buffer: 250 ml 0.2 M KH_2PO_4 (27.22 g/liter), 197 ml 0.2 M NaOH (8.0 g NaOH/liter) and distilled water to 1 liter.

4. 2.4% hydrogen peroxide: 1 cc 30% H_2O_2 + 11.5 cc phosphate buffer. Must be prepared fresh and exact.

Procedure:

1. To a graduated centrifuge tube containing 4.0 ml of citrate-saline solution, add 0.4 ml of mixed whole blood.

2. Centrifuge 10 minutes at 1000 rpm.

3. Discard supernatant.

4. Add saline buffer mixture of equal parts of 0.9% saline and phosphate buffer to bring the volume to 8.0 ml.

5. Incubate at 37° for 15 minutes.

6. Centrifuge and discard supernatant.

7. Add 0.9% saline to bring the volume to 4.0 ml and shake gently to ensure a uniform suspension.

8. Into each of 5 small round-bottom test tubes, pipette 0.50 ml of suspension.

9. Into 4 of the 5 tubes (tubes B, C, D, E) add 0.50 ml of buffered 2.4% H_2O_2, and into the fifth tube (tube A) pipette 0.50 ml phosphate buffer.

10. Incubate all tubes at 37° C for 15 minutes.

11. Shake gently for 2-3/4 hours at room temperature.

12. At the end of the 3-hour period, add 9.0 ml saline buffer to the control (tube A) and 3 of the treatment tubes containing H_2O_2 (tubes B, C, D). To the fourth tube containing peroxide (tube E), add 9.0 ml of distilled water.

13. Mix tubes by inversion and centrifuge.

14. Read supernatant at 540 mμ after setting instrument at 0 OD with blank or control tube.

Calculations:

$$\% \text{ hemolysis} = 100 \times \frac{(\text{OD tubes B + C + D})/3}{\text{OD tube E}}$$

Comment: This procedure is valid only for freshly drawn blood samples. Only blood samples less than 1 hour old should be used.

Interpretation: The following values are arbitrarily used in interpreting results:

10 %	-	normal
10-20%	-	marginal or low
20%	-	deficient

PREPARATION OF DOWEX-50

Reference: J. Biol. Chem. 211: 117.

Dowex-50 (12% cross-linkage, 200-400 mesh) is sedimented in H_2O and the very fine and very coarse particles are removed by decantation. 1500 ml of the packed resin in a column are washed with 25 liters of 8 N HCl, 12 liters of 4 N HCl and 10 liters of distilled water at a flow rate of 750 ml per hour.

COLLECTION OF FRACTIONS A THROUGH E

Reference: J. Biol. Chem. 219: 988.

Diazotizable amines in urine can be resolved into five fractions on a column of Dowex-50. Hand-operated columns were developed for the separation of these fractions.

One per cent of a 24-hour specimen of human urine was acidified with 4.0 ml of 1.0 N HCl and diluted to 40 ml. To determine the recovery of compounds from the columns, two similar samples were prepared. To one were added: 400 γ of o-aminohippuric acid and 400 γ of kynurenine sulfate; to the other were added 400 γ of acetylkynurenine.

The samples were then applied to 10 cm columns of Dowex-50 and passed through at a flow rate of 12-20 drops per minute. The samples were followed by two 20 ml washes of 0.1 N HCl. The combined filtrate and washes (80 ml)

Frac. A	.1 N HCl - two 20 ml washings
B	.5 N HCl - 80 ml
C	1.0 N HCl - 80 ml
D	2.4 N HCl - 80 ml
E	5.0 N HCl - 80 ml

constitute fraction A, the identity of which has not been established. The columns were then washed with 80 ml of 0.5 N HCl, which gives fraction B which contains anthranilic acid glucuronide when present. Fraction C, eluted by 80 ml of 1.0 N γ HCl, contained o-aminohippuric acid. Anthranilic acid and acetylkynurenine

Collection of Fractions A through E

were eluted together in fraction D with 80 ml of 2.4 N HCl, while 3-hydroxykynurenine was washed off the column in fraction E with 80 ml of 5.0 N HCl. The other known components of fraction E which include kynurenine, kynurenic acid and xanthurenic acid do not interfere with the determination of 3-hydroxykynurenine.

The columns can be reused after regeneration with 125 ml of 8 N HCl followed by 125 ml of water. Immediately before use, the columns were washed with about 50 ml of water.

DETERMINATION OF 3-HYDROXYKYNURENINE

Reference: J. Biol. Chem. 227: 649.

Fraction E is obtained as described above. To determine the recovery of 3-hydroxykynurenine in the column, an additional column is used to which is applied a duplicate urine sample with 400-800 γ of hydroxykynurenine. 3.0 ml of fraction E is pipetted into each of three colorimeter tubes, and 0.2 ml of 0.25% $NaNO_2$ is added to tubes 2 and 3. Three minutes after mixing the nitrite, 0.2 ml of 10% ammonium sulfamate is added to all the tubes. The first tube is then made to the same volume as the others by the addition of 0.2 ml of H_2O. The OD of 367 mu is measured in a Beckman DU spectrophotometer by using the first tube as a blank. A standard curve is run at the same time with hydroxykynurenine in 5 N HCl (10-40 γ/tube). From the standard curve, the column recovery and the mg/24 hours is calculated.

ASCORBIC ACID

Blood:

*1. 15 ml of 6% TCA in beaker.

*2. Add 5 ml of blood to TCA, mix and filter into another beaker using #1 filter paper.

3. Add approximately 1/2 teaspoon of Norit charcoal, mix and filter into another beaker using #42 filter paper.

4. Place 4 ml of filtrate in each of two cuvettes (2 tubes/sample).

5. Add 1 drop of thiourea and 1 ml of DNPH to each tube.

6. Boil for 15 minutes.

*Change to 2.5 cc blood and 17.5 cc 6% TCA if O.D. readings become too high.

Urine:

1. 19 ml of 4% TCA in beaker.

2. 1 ml of urine to TCA, mix, add Norit and filter using #42 filter paper.

3. Follow steps 4, 5, and 6 of directions for blood.

Standard:

1. 5 ml of working standard in beaker, add charcoal and filter using #42 filter paper.

2. 18 ml of 4% TCA in beaker, add charcoal and filter using #42 filter paper.

3. Place 1 ml of working standard and 3 ml of 4% TCA in each of two cuvettes.

4. Add 1 drop of thiourea and 1 ml of DNPH to each tube.

5. Boil for 15 minutes.

Blank:

1. 4 ml of 4% TCA used in Step 2 of Standard in a cuvette.

2. Boil for 15 minutes.

3. Add 1 ml of DNPH after 85% H_2SO_4 has been added.

All Tubes:

1. Cool in ice water.

2. Add 5 ml of 85% H_2SO_4 and mix well.

Ascorbic Acid

Calculations:

Blood (5.0 ml) unk/std x 2 = mgm ascorbate/100 ml of blood.

(2.5 ml) unk/std x 4 = mgm ascorbate/100 ml of blood.

Urine (1.0 ml) unk/std x .1 x TV = mgm ascorbate/24 hr.

Reagents:

1. Stock ascorbic acid standard: Accurately weigh exactly 100 mg of 1-ascorbic acid (Eastman) and place in a 100 ml volume tric flask. Dilute to volume with 4% trichloroacetic acid solution.

2. Working ascorbic acid standard: Dilute 2 ml of stock standard to 100 ml with 4% trichloroacetic acid. 1 ml = 20 gamma of 1-ascorbic acid.

3. Trichloroacetic acid: 4% solution; 6% solution. Use reagent grade.

4. 2,4-dinitrophenylhydrazine: 2 gm of 2,4-dinitrophenylhydrazine (Eastman) are dissolved in 100 ml of 9N sulfuric acid. Let stand overnight and filter through Whatman No. 42 filter paper.

5. 85% sulfuric acid: to 100 ml of water add 900 ml of concentrated sulfuric acid. Use reagent grade.

6. Thiourea: Dissolve 10 gm of thiourea (Eastman) in 50 ml of absolute ethyl alcohol and dilute to 100 ml with distilled water. This reagent should keep 2 months.

7. Norit: Use U.S.P. grade. If blanks read too high, add 1 liter of 10% hydrochloric acid to 200 gm of Norit in a large flask, bring to a boil, and filter with suction. Stir the cake with 1 liter of water and again filter. Dry the cake in an oven overnight at 100-120°.

5-HYDROXYINDOLEACETIC ACID

References:

1. Pierce, Carole. Assay and importance of serotonin and its metabolites. Am. J. Clin. Path. 30: 230-233, 1958.

2. Ross, George I., Bernard Weinstein and Bernard Kabakow. The influence of phenothiazine and some of its derivatives on the determination of 5-hydroxyindoleacetic acid in urine. Clin. Chem. 4: 66-76, 1958.

Obtain a 24-hour specimen of urine, to which has been added 3 ml of toluene and 25 ml of glacial acetic acid. The pH must be maintained below 5 at all times. Patients should be off all drugs for 4 days prior to collection of urine. Chlorpromazine and other drugs may interfere with the determination.

Procedure:

	1	2	3
Filtered urine	1.0	1.0	1.0
5-hydroxyindoleacetic acid (50 mg/L)	-	1.0	-
Distilled water	4.0	3.0	4.0
1-Nitroso-2 naphthol reagent	2.5	2.5	-
Sulfuric acid 2.0 N	-	-	2.5
Nitrous acid reagent	2.5	2.5	-

Mix thoroughly and allow to set at room temperature for 10 minutes. Add 10 ml of chloroform to each tube and shake vigorously. Centrifuge, separate lower chloroform layers and discard. Add another 10 ml of chloroform, shake vigorously, centrifuge and discard lower chloroform layers. Read density of tube #1 and #2, in 19 x 150 mm cuvettes, using contents of tube #3 to obtain 0 density in spectrophotometer at 540 mμ.

Calculation:

$$\frac{D_1}{D_2 - D_1} \times 50 = \text{Mg 5-hydroxyindoleacetic acid/liter}$$

5-Hydroxyindoleacetic acid

Normals:

Less than 8.0 mg of 5-hydroxyindoleacetic acid/24 hours.

Reagents:

1. 1-Nitroso-2 naphthol reagent: 0.1% in 95% ethyl alcohol.

2. Nitrous acid reagent: Prepare immediately before use by adding 0.2 ml of 2.5% sodium nitrite to 5.0 ml of 2.0 N H_2SO_4.

3. Chloroform or ethylene dichloride.

4. 5-hydroxyindoleacetic acid standard: Dissolve 25 mg of the acid in 500 ml of distilled water. Add a few drops of glacial acetic acid to keep pH below 3.0.

THE ESTIMATION OF 5-HYDROXYTRYPTAMINE

Reference. J.B.C., vol. 215, Jul-Aug 1955

Materials. 5-hydroxytryptamine was made available by The Upjohn Company and the Abbott Laboratories as the creatinine sulfate complex.

Reagents:

1. Borate buffer: To 94.2 gm of boric acid dissolved in 3 liters of water are added 165 ml of 10 N NaOH. The buffer solution is then saturated with n-butanol and NaCl by adding these substances in excess and shaking. Excess n-butanol is removed by aspiration and excess salt is permitted to settle. The final pH should be approximately 10.

2. n-butanol: Reagent grade butanol is purified by shaking first with an equal volume of 0.1 N NaOH, then with an equal volume of 0.1 N HCl and finally twice with distilled water

3. Heptane: Practical grade of heptane is treated in the same manner as the n-butanol.

4. 1-nitroso-2-naphthol reagent: 0.1% 1-nitroso-2-naphthol in 95% ethyl alcohol.

5. Nitrous acid reagent: To 5 ml of 2 N H_2SO_4 is added 0.2 ml of 2.5% $NaNO_2$. The reagent should be prepared fresh daily.

Procedure for Extraction of 5HTA from Tissue Extracts:

5HTA can be extracted into n-butanol from salt-saturated solutions buffered to pH 10. By washing the n-butanol several times with salt-saturated buffer of pH 10, substances such as 5-hydroxytryptophan, tryptophan and normally occurring "blank" impurities can be removed. Addition of heptane to the n-butanol makes it possible to reextract the 5HTA into aqueous acid, in which it can be assayed. 3 ml of tissue extract are adjusted to approximately pH 10 and transferred to a 60 ml glass-stoppered bottle containing 1 ml of the borate buffer, 2 gm of NaCl and 15 ml of n-butanol. The bottle is shaken for 5 minutes. The contents are decanted into a 45 ml glass-stoppered centrifuge tube and centrifuged. The aqueous phase is removed by aspiration 15 ml of borate buffer reagent are added, and the tube is shaken for 5 minutes.

The Estimation of 5-Hydroxytryptamine

After allowing the phases to separate, the aqueous layer is removed, replaced with 15 ml of fresh buffer reagent, shaken for 5 minutes and centrifuged. A 10 ml aliquot of the butanol layer is transferred to another 45 ml glass-stoppered centrifuge tube containing 20 ml of heptane and 3 ml of 0.5 M formate buffer of pH 1.[1] The tube is shaken, centrifuged and the supernatant solvent layer is removed by aspiration. The aqueous layer containing the extracted 5HTA is assayed by one of the three methods presented below.

The distribution of 5HTA between the salt-saturated buffer, pH 10, and n-butanol is such that with the volumes used only about 95% is extracted into the organic solvent. Since three equilibrations of the butanol with salt-saturated buffer are employed, the final recovery of 5HTA is about 85%. Standards are, therefore, prepared by carrying known amounts of 5HTA through the entire extraction procedure.

The extraction procedure yields extracts which are essentially free of any interfering material, within the limits of sensitivity found for each of the following analytical procedures. The extraction procedure also removes compounds such as tryptophan and 5-hydroxytryptophan when they are used as substrates for the production of 5HTA.

Colorimetric Procedure:

To 2 ml of the acid extract containing 0.05 to 0.8 μmole of 5HTA in a glass-stoppered centrifuge tube is added 1 ml each of the nitroso-naphthol and the acid nitrite reagents. The tube is stoppered, shaken and placed in a water bath at 55° for 5 minutes. 10 ml of ethylene dichloride are added, and the tube is shaken to extract the unchanged nitrosonaphthol. The tube is then centrifuged at low speed, and the supernatant aqueous layer is transferred to a cuvette. When measured at 540 mμ in a Beckman spectrophotometer, optical density is proportional to concentrations up to 0.8 μmole. The color is stable, less than 1% change occurring on standing 1 hour.

[1]When the colorimetric procedure is to be employed, 0.2 M HCl is used instead of formate buffer.

5-HYDROXY-3-INDOLEACETIC ACID ASSAY IN URINE

Reference: J.B.C., vol. 216, Sep-Oct 1955

Reagents:

1. 1-Nitroso-2-naphthol. 0.1% solution in ethanol.

2. Nitrous acid reagent. To 5 ml of 2 N H_2SO_4 is added 0.2 ml of 2.5% $NaNO_2$. The reagent should be prepared fresh daily.

3. Ethyl ether, reagent grade, was washed once with a dilute solution of ferrous sulfate to destroy peroxides and twice with water.

4. 0.5% 2,4-dinitrophenylhydrazine in 2 N HCl.

5. 0.5 M phosphate buffer, pH 7.0.

Identification of 5HIAA in Human Urine: In a typical experiment, 90 ml of urine were adjusted to about pH 3 by the dropwise addition of concentrated HCl and extracted twice with 400 ml portions of $CHCl_3$ to remove indoleacetic acid. The $CHCl_3$ was discarded and the acidified urine after saturation with NaCl was extracted with 250 ml of ether. The apparent 5HIAA was returned to an aqueous phase by shaking the ether solution with 50 ml of phosphate buffer, pH 7.0. The latter phase was acidified and extracted with 250 ml of fresh ether.

Determination of 5HIAA in Urine: The procedure involves preliminary treatment of the urine with dinitrophenylhydrazine to react with any keto acids present, since these substances when extracted through the procedure interfere with the final color. Normally, the interference is slight, but in certain metabolic disorders, when large amounts of keto acids are excreted, their removal is essential. Extraction with $CHCl_3$ then removes indoleacetic acid, which gives a slight color with nitrous acid. The low distribution coefficient of 5HIAA between ether and water makes it necessary to saturate the aqueous phase with NaCl to extract it into ether. The 5HIAA is returned to buffer of pH 7.0 for colorimetric assay. At pH values above this, the compound becomes progressively more unstable.

5-Hydroxy-3-Indoleacetic Acid Assay in Urine

To 6 ml of urine in a 50 ml glass-stoppered bottle are added 6 ml of 2,4-dinitrophenylhydrazine reagent.[1] After 30 minutes, 25 ml of $CHCl_3$ are added, and the bottle is shaken for a few minutes and then centrifuged. The organic layer is removed, replaced with a fresh 25 ml portion of $CHCl_3$ and the extraction repeated. After centrifuging, a 20 ml aliquot of the aqueous layer is transferred to a 40 ml glass-stoppered centrifuge tube containing about 4 gm of NaCl and 25 ml of ether. The tube is shaken for 5 minutes. Following centrifugation, a 20 ml aliquot of the ether is transferred to another 40 ml glass-stoppered centrifuge tube containing 1.5 ml of buffer at pH 7.0. The tube is shaken for 5 minutes, centrifuged and the ether layer removed by aspiration. 1 ml of the aqueous phase is transferred to a 15 ml glass-stoppered centrifuge tube containing 0.5 ml of nitrosonaphthol reagent. Following this, 0.5 ml of nitrous acid reagent is added, and the sample is then mixed well and warmed at 37° for 5 minutes. 5 ml of ethyl acetate are then added and the tube is shaken. After separation of the phases and removal of the ethyl acetate by aspiration, a second 5 ml portion of ethyl acetate is added, and this step is repeated. The final aqueous layer is transferred to a micro cuvette, and the optical density is measured at 540 mμ.[2]

Standards are prepared by treating 6 ml of solution containing 10 to 200γ of 5HIAA exactly as for the urine samples. The reagent blank used for the blank setting of the instrument is prepared by treating 6 ml of water in the same manner.

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[1]Some urines yield a large precipitate at this step. This should be removed by centrifugation before continuing the assay.

[2]The micro cell adapter of the Beckman spectrophotometer has been used for these measurements.

XANTHURENIC ACID TEST

Reference: Manual for Nutrition Surveys, Interdepartmental Committee on Nutrition for National Defense, May 1958, p. 88.

Load Test: A preliminary 24-hour urine collection is made. Toluene or oxalic acid may be used as preservative. The subject is then given 10 gm of DL-Tryptophan (or 5 gm of L-Tryptophan) in water and another 24-hour urine collected. Meals may be taken as usual.

Analysis for Xanthurenic Acid:

1. Reagents:

a. 0.4 M tris (hydroxymethyl) amino methane buffer, pH 7.8: dissolve 58 gm of maleic acid (C.P.) and 60.6 gm of tris in 500 ml H_2O. About 4 gm charcoal is added. The mixture is then shaken and, after standing 10 minutes, is filtered. 48.4 ml of 1 N NaOH is added to 40 ml of the filtrate and, after diluting to 100 ml with H_2O, the pH is checked and adjusted if necessary. Buffer is stable in icebox.

b. 1.7% Fe $NH_4(SO_4)_2 \cdot 12H_2O$ (0.85 gm in 50 ml H_2O).

c. 60 mg pure xanthurenic acid (XA) in 180 ml ethanol. The XA is brought into solution by drop-wise addition of 1 N NH_4OH.

2. Procedure:

a. Place 5 ml of filtered urine and 5 ml H_2O in a test tube. More urine can be used if only small amounts of XA are present.

b. Add 10 ml buffer and mix by inversion.

c. Pipette 10 ml to a cuvette marked "unknown" and pour the remainder to another cuvette marked "blank."

d. To the "unknown" add 0.1 ml of 1.7% Fe $NH_4(SO_4)_2$ and shake.

e. Let stand 5 minutes.

f. Set the colorimeter at 610 mμ; adjust to zero O.D. with the "blank." Read unknown solution.

3. Calculation: From the standard curve, calculate the mg xanthurenic acid excreted in 24 hours.

Xanthurenic Acid Test

4. Comments: Urine preserved with toluene can be kept in the icebox without loss of XA. The color of XA with the reagent is stable for several hours. Although pyridoxine deficiency has never been reported in adults under normal feeding conditions, the tryptophan load test would appear to be indicated in cases where other B-vitamins are known to be deficient. If B_6 deficiency is indicated as a result of the tryptophan load test, the oral administration of 15 mg pyridoxine daily for 7 days should reduce the xanthurenic acid excretion, following tryptophan, to almost normal amounts.

5. References:

a. Vilter et al. J. Lab. Clin. Med. 42: 335, 1953.

b. Greenburg et al. Arch. Biochem. 21: 237, 1949.

c. Wachstein and Gudaitis. Am. J. Clin. Path. 22: 652, 1952.

OXALATE DETERMINATION

<u>Reference</u>: Archer, H. E., A. E. Dormer, E. F. Scowen and R. W. E. Watts. Studies on the urinary excretion of oxalate by normal subjects. Clin. Sci. 16: 405, 1957.

<u>Reagents</u>:

1. 0.35 N NH_4OH: 22 ml NH_4OH + 978 ml H_2O.
2. 0.04% Bromcresol purple : 0.4 gm/liter.
3. 6 N HOAc : 345 ml HOAc + 655 ml H_2O.
4. 5% $CaCl_2$: 50 gm $CaCl_2$ in 1 liter H_2O.
5. Standard: 1 gm sodium oxalate diluted to 500 ml with H_2O.
6. $KMnO_4$: 0.6 gm $KMnO_4$ diluted to 2 liters with H_2O.
7. 1 N H_2SO_4 : 29 ml H_2SO_4 + 971 ml H_2O.

<u>Calculations</u>:

Standard:

Corrects for use of Na oxalate as standard since all unknowns are precipitated as Ca oxalate

$$\frac{\text{Mg Na oxalate}}{\text{Vol. of } KMnO_4 \text{ used (ml in test tube)}} \times 0.965 = \text{Factor}$$

Unknowns:

$$\text{(ml } KMnO_4 \text{ used) (in titrating)} \times \text{factor} \times \frac{\text{total vol. of unknown}}{\text{vol. used in isolation}} = \text{mg/total vol.}$$

5 ml of standard titrates to ~16.2

$$\frac{10.0}{16.2} \times .965 = \text{factor}$$

<u>Procedure</u>:

1. Pour 37 ml urine in centrifuge tubes.
2. Pour urine into 100 ml beakers.
3. Add 0.8 ml of Bromcresol purple to each sample.
4. Add NH_4OH until purple color appears (~pH 8.5-9.0).

Oxalate Determination

5. Adjust pH to 5.1 with 6 N HOAc.

6. Add 2 ml of 5% $CaCl_2$ to each tube (beaker).

7. Pour samples back into centrifuge tubes and let stand for 18 hours.

8. Centrifuge for 20 minutes at 2700 rpm.

9. Siphon off supernatant and then add 4 ml 0.35 N NH_4OH and vortex; then add 4 ml more of 0.35 N NH_4OH.

10. Pour contents into 15 ml centrifuge tubes and repeat step 8.

11. Siphon off supernatant and then add 4 ml 0.35 N NH_4OH and vortex; then add 4 ml more of 0.35 N NH_4OH and repeat step 8.

12. Siphon off supernatant and add 1 N H_2SO_4 (2 ml) and mix.

13. Titrate using $KMnO_4$.

PARA-AMINO SALICYLIC ACID DETERMINATION IN BODY FLUIDS

E. K. Marshall, Jr. Method:

References:

1. Marshall, E. K., Jr. Proc. Soc. Exp. Biol. Med. 68: 471, 1948.

2. Bratton, A. C. and E. K. Marshall, Jr. J. Biol. Chem. 128: 537, 1939.

Reagents:

1. p-Toluene Sulfonic Acid, 20%: 100 grams dissolved in distd-demin water and made up to 500 ml final volume. Note

2. p-Toluene Sulfonic Acid, 4%: 20 ml of the above 20% solution made up to a final volume of 100 ml with distd-demin water.

3. Hydrochloric Acid, 6N.

4. Sodium Nitrite: 0.025 grams dissolved in a total volume of 25 ml with distd-demin water. Prepare fresh daily.

5. Ammonium Sulfamate: 0.125 grams dissolved in a total volume of 25 ml with distd-demin water.

6. Bratton-Marshall Reagent: 0.025 grams of N-(1-naphthyl)-ethylenediamine dihydrochloride dissolved in a total volume of 25 ml with distd-demin water. Store in an amber bottle in the refrigerator. Note 2.

Preparation of the Sample:

1. Blood: Pipette 1 ml of the blood plasma or serum into a 50-ml Erlenmeyer flask containing 31 ml of distd-demin water. Slowly add with shaking 8 ml of 20% toluene sulfonic acid. Shake well and filter through a hard paper such as Whatman #50. The filtrate should be perfectly clear. If not, refilter.

2. Urine: Since the concentration of the PAS is much greater than in the blood, preliminary dilution is necessary; usually a 1:1000 dilution. A greater dilution may be necessary.

3. Spinal Fluid: Treat in the same manner as the plasma or serum.

Para-Amino Salicylic Acid Determination in Body Fluids

Standard Solutions:

1. Master Standard: A master standard solution is made up by dissolving, without the use of heat, 100 milligrams of pure PAS or 137.89 milligrams of pure NaPAS.$2H_2O$ in a total volume of 1000 ml of distd-demin water (equals 10 mg%). A small amount of sodium bicarbonate may be used to facilitate the solution of the PAS. The NaPAS.$2H_2O$ is readily soluble without the use of sodium bicarbonate.

2. Working Standard: To prepare working standards, dilute the master standard 5 ml to 100 ml, 20 ml of this being 20% toluene sulfonic acid. From the diluted standard, make up the following series of dilutions:

Tube #	Dilute Standard	4% Toluene Sulfonic Acid (ml)	Final PAS conc mg%
1	0	5	0
2	1	4	0.1
3	2	3	0.2
4	3	2	0.3
5	4	1	0.4
6	5	0	0.5

For the preparation of a standard curve, follow the above series of dilutions through the procedure below.

Sample Procedure:

1. To 5 ml of the PAS filtrates in 16 x 150 mm test tubes (made up in 4% toluene sulfonic acid) add 1 ml 6 N HCl. Cool in an ice-water bath to 1° C and add 0.5 ml of nitrite reagent. Mix and let stand 3 minutes. Then, add 0.5 ml of the ammonium sulfamate solution. Remove immediately from the ice bath and mix After 2 minutes, add 0.5 ml of the Bratton-Marshall reagent and mix thoroughly Prepare at the same time a reagent blank consisting of 5 ml of 4% toluene sulfonic acid and carry through the procedure as for samples. Allow samples and reagent

blank to stand in the dark for 20 minutes to allow the color to develop fully. Transfer samples and reagent blank to clean, dry 19 x 150 mm cuvettes.

2. Read on the Beckman Model B at 540 mμ.

Calculations: From the reading of the unknown taken from the standard curve, multiply by the dilution factor of 40 and this gives the PAS as mg%. To convert to micrograms (mcg or γ)/ml multiply mg% by 10.

Notes:

1. Filter the 20% toluene sulfonic acid once, using a Buchner funnel and two Whatman #42 filter papers and again a Buchner funnel with Whatman #50 paper. Distd-demin water refers to distilled-demineralized water.

2. In the purification of N-(1-naphthyl)-ethylenediamine dihydrochloride, for each 3 grams of the Eastman product, add 25 ml distd-demin water, add a small quantity of activated charcoal, Norite or Darco, heat to near boiling, filter and measure the volume of the filtrate. Add an equal volume of concentrated HCl, shake and place in freezer. Stir occasionally. It may be necessary to scratch the inside of the flask to induce crystallization. Filter over a Buchner funnel with #42 Whatman. Place the precipitate in a 125-ml Erlenmeyer flask, add 20 ml water, heat to near boiling. Add activated charcoal, if necessary. If no charcoal is added, place 20 ml concentrated HCl in flask. Agitate flask, place in freezer to allow crystals to form. Filter over Buchner with #42 Whatman, wash precipitate with a small amount of acetone, allow to dry in the air. Place the precipitate in 110° oven for a short while, transfer while still warm to a vacuum desiccator.

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